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(54) Title: METHOD FOR ISOLATING HEPATITIS C VIRUS PEPTIDES

(57) Abstract: Described is a method for isolating Hepatitis C Virus peptides (HPs) which have a binding capacity to a MHC/HLA molecule or a complex comprising said HCV-peptide and said MHC/HLA molecule characterized by the following steps: - providing a pool of HCV-peptide, said pool containing HCV-peptides which bind to said MHC/HLA molecule and HCV-peptides which do not bind to said MHC/HLA molecule, -contacting said MHC/HLA molecule with said pool of HCV-peptides whereby a HCV-peptide which has a binding capacity to said MHC/HLA molecule binds to said MHC/HLA molecule and a complex comprising said HCV-peptide and said MHC/HLA molecule is formed, -detecting and optionally separating said complex from the HCV-peptide which do not bind to said MHC/HLA molecule and optionally isolating and characterising the HCV-peptide from said complex.

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Method for Isolating Hepatitis C Virus Peptides

The present invention relates to a method for isolating HCV-peptides, especially for isolating HCV T cell epitopes which have a binding capacity to a MHC/HLA molecule.

The immune system is a complex network of inter-related cell types and molecules, which has evolved in order to protect multicellular organisms from infectious microorganisms. It can be divided into the evolutionary older innate (or natural) immunity and adaptive (or acquired) immunity. The innate immune system recognizes patterns, which are usually common and essential for pathogens. For this limited number of molecular structures germline encoded receptors have evolved. By contrast, cells of the adaptive immune system - B and T lymphocytes - can recognize a huge variety of antigenic structures. The receptors, termed according to the cell types expressing them, B cell receptor (BCR, its soluble versions are called antibodies) and T cell receptor (TCR, only cell-surface associated forms) are generated by somatic recombination and show a clonal distribution. Thus, initially there is only small number of cells with a certain specificity. Upon antigen encounter these cells start to divide (clonal expansion) to generate an effector population able to cope with the antigen. After elimination of antigen a specialized sub-population of cells specifically recognizing this antigen remains as immunological memory. Taken together the adaptive immune system is slow (compared to innate immunity), however specific and it improves upon repeated exposure to a given pathogen/antigen.

T cells have a central role in adaptive immunity. Their receptors (TCRs) recognize "major histocompatibility complex" (MHC or HLA):peptide complexes on the surface of cells. These peptides are called T cell epitopes and represent degradation products of antigens. There are two major classes of T cells: CD8-positive cytotoxic T cells (CTL) are restricted to MHC class I. CD4-positive helper T cells (HTL) are restricted to MHC class II. HTL are essential for many features of adaptive immunity: activation of so called "professional antigen-presenting cells" (APCs), immunoglobulin (Ig) class switch, the germinal center reaction and

Ig affinity maturation, activation of CTL, immunological memory, regulation of the immune response and others.

MHC molecules collect peptides inside the cell and present them on the cell surface to TCRs of T cells. There are two major classes of MHC, class I recognized by CD8-positive CTL and class II recognized by CD4-positive HTL.

MHC class I molecules consist of a membrane-anchored alpha-chain of 45 kDa and the non-covalently attached β 2-microglobulin (β 2m) of 12 kDa. Resolution of the 3-dimensional structure by X-ray crystallography (Stern and Wiley 1994) revealed that the alpha-chain possesses a cleft, which is closed at both ends and accommodates peptides from 8 to 11 amino acids length. Class I molecules are ubiquitously expressed, and the peptides they present originate from cytoplasmic proteins. These are degraded by the proteasome, and the resulting peptides are actively transported into the endoplasmatic reticulum (ER). There, with the help of several chaperones, MHC:peptide complexes are formed and transported to the cell surface (Heemels 1995). Thus, MHC class I mirrors the proteome of a cell on its surface and allows T cells to recognize intracellular pathogens or malignant cells.

MHC class II molecules consist of two membrane-anchored proteins (alpha- and beta-chain) of 35 kDa and 30 kDa, respectively. These together form a cleft, open at both ends, which can accommodate peptides of variable length, usually from 12 to 25 amino acids. Despite these differences, class I and II molecules share surprising structural similarity (Stern and Wiley 1994). Class II molecules are only expressed on professional APC including dendritic cells (DC), B-cells and macrophages/monocytes. These cells are specialized in taking up and processing antigens in the endosomal pathway. Immediately after their biosynthesis, class II molecules are complexed by the so-called invariant chain (Ii), which prevents binding of peptides in the ER. When vesicles containing class II:Ii complexes fuse with endosomes containing degradation products of exogenous antigen, Ii is degraded until the MHC binding cleft is only complexed by the so-called CLIP peptide. The latter is with the help of chaperones like HLA-DM exchanged by antigenic peptides (Villadangos 2000).

Finally, MHC:peptide complexes are again presented on the surface of APCs, which interact in numerous ways with HTL.

Being both polygenic and extremely polymorphic, the MHC system is highly complex. For the class I alpha-chain in humans there are three gene loci termed HLA-A, -B and -C. Likewise, there are three class II alpha-chain loci (DRA, DQA, DPA); for class II beta-chain loci the situation is even more complex as there are four different DR beta-chains (DRB1,2,3,5) plus DQB and DPB. Except the monomorphic DR alpha-chain DRA, each gene locus is present in many different alleles (dozens to hundreds) in the population (Klein 1986). Different alleles have largely distinct binding specificities for peptides. Alleles are designated, for example, HLA-A*0201 or HLA-DRB1*0401 or HLA-DPA*0101/DPB*0401.

T cell epitopes have been identified by a variety of approaches (Van den Eynde 1997). T cell lines and clones have for instance been used to screen cDNA expression libraries for instance in the context of COS cells transfected with the appropriate HLA-molecule. Alternatively, biochemical approaches have been pursued. The latter involved elution of natural ligands from MHC molecules on the surface of target cells, the separation of these peptides by several chromatography steps, analysis of their reactivity with lymphocytes in epitope reconstitution assays and sequencing by mass spectrometry (Wölfel et al. 1994, Cox et al. 1994).

Recently the advent of highly sensitive cytokine detection assays like the IFN-gamma ELISpot allowed using lymphocytes directly ex vivo for screening of overlapping synthetic peptides (Maecker 2001, Kern 2000, Tobery 2001). Primarily, Kern et al. (1999&2000) used arrays of pools of overlapping 9mer peptides to map CD8+ T cell epitopes in vitro. Later, Tobery et al., 2001 modified this approach and demonstrated that pools containing as many as 64 20mer peptides may be used to screen for both CD8+ and CD4+ T cell epitopes in mice. Both these methods were based on the monitoring of antigen-specific response by measuring INF-gamma production either by intracellular staining (Kern et al 2000) or in ELISpot assay (Tobery et al., 2001). By use of mixtures of 15-mers the CD4+ T cell responses are approximately

equal to those detected when whole soluble protein was used as an antigen, while -not surprising- the CD8+ T cell responses are significantly higher than the often negligible responses detected with soluble protein stimulation. Furthermore, the CD8+ T cell responses to a mixture of 15 amino acid peptides are similar to those obtained with a mix of 8-12 amino acid peptides, selected to represent known MHC class I minimal epitopes. Most probably peptidases associated with the cell membrane are responsible for "clipping" peptides to optimal length under these circumstances (Maecker et al, 2001).

An interesting alternative is to screen synthetic combinatorial peptide libraries with specific lymphocytes. For instance, a decapeptide library consisting of 200 mixtures arranged in a positional scanning format, has been successfully used for identification of peptide ligands that stimulate clonotypic populations of T cells (Wilson, et al., J. Immunol., 1999, 163:6424-6434).

Many T cell epitopes have been identified by so called "Reverse immunological approaches" (Rammensee 1999). In this case the protein giving rise to a potential T cell epitope is known, and its primary sequence is scanned for HLA binding motifs. Typically dozens to hundreds of candidate peptides or even a full set of overlapping peptides are synthesized and tested for binding to HLA molecules. Usually, the best binders are selected for further characterization with regard to their reactivity with T cells. This can for instance be done by priming T cells in vitro or in vivo with the help of HLA transgenic mice.

Hepatitis C Virus (HCV) is a member of the flaviviridae chronically infecting about 170 million people worldwide. There are at least 6 HCV genotypes and more than 50 subtypes have been described. In America, Europe and Japan genotypes 1, 2 and 3 are most common. The geographic distribution of HCV genotypes varies greatly with genotype 1a being predominant in the USA and parts of Western Europe, whereas 1b predominates in Southern and Central Europe (Bellentani 2000).

HCV is transmitted through the parenteral or percutan route, and

replicates in hepatocytes. About 15% of patients experience acute self-limited hepatitis associated with viral clearance and recovery. About 80% of infected persons become chronic carriers. Infection often persists asymptomatically with slow progression for years, however ultimately HCV is a major cause of cirrhosis, end-stage liver disease and liver cancer (Liang 2000). Strength and quality of both HTL and CTL responses determine whether patients recover (spontaneously or as a consequence of therapy) or develop chronic infection (Liang 2000).

Standard therapy of HCV comprises a combination of pegylated interferon-alpha and the antiviral ribavirin. Virologic responses are, depending on the genotype, achieved in about 50% of HCV patients. The low tolerability and the considerable side effects of this therapy clearly necessitate novel therapeutic intervention including therapeutic vaccines (Cornberg 2002). However, presently the detailed understanding of which epitopes in which MHC combination lead to successful immune responses is lacking (Ward 2002). Therefore, a comprehensive analysis of the T-cell response against the entire HCV is required for development of therapeutic epitope-based vaccines.

The HCV virion contains a 9.5-kilobase positive single-strand RNA genome encoding a large single polyprotein of about 3000 amino acids. The latter is processed to at least 10 proteins by both host and HCV-encoded proteolytic activities (Liang 2000). Importantly, the HCV RNA-dependent RNA polymerase is error prone giving rise to the evolution of viral quasispecies and contributing to immune-escape variants (Farci 2000).

It is an object of the present invention to provide a method for screening HCV-peptides for specific MHC molecules, preferably for delivering suitable and specific HCV T cell epitopes selected from a variety of HCV-peptides having unknown specificity for a given MHC molecule and thereby to provide efficient means for preventing and combatting HCV infections.

Therefore the present invention provides a method for isolating HCV-peptides which have a binding capacity to a MHC/HLA molecule or a complex comprising said HCV-peptide and said MHC/HLA mo-

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lecule which method comprises the following steps:

- providing a pool of HCV-peptides, said pool containing HCV-peptides which bind to said MHC/HLA molecule and HCV-peptides which do not bind to said MHC/HLA molecule,
- contacting said MHC/HLA molecule with said pool of HCV-peptides whereby a HCV-peptide which has a binding capacity to said MHC/HLA molecule binds to said MHC/HLA molecule and a complex comprising said HCV-peptide and said MHC/HLA molecule is formed,
- detecting and optionally separating said complex from the HCV-peptides which do not bind to said MHC/HLA molecule and
- optionally isolating and characterising the HCV-peptide from said complex.

The present invention also provides a method for isolating HCV T cell epitopes which have a binding capacity to a MHC/HLA molecule or a complex comprising said epitope and said MHC/HLA molecule which method comprises the following steps:

- providing a pool of HCV-peptides, said pool containing HCV-peptides which bind to a MHC/HLA molecule and HCV-peptides which do not bind to said MHC/HLA molecule,
- contacting said MHC/HLA molecule with said pool of HCV-peptides whereby a HCV-peptide which has a binding capacity to said MHC/HLA molecule binds to said MHC/HLA molecule and a complex comprising said HCV-peptide and said MHC/HLA molecule is formed,
- detecting and optionally separating said complex from the HCV-peptides which do not bind to said MHC/HLA molecule,
- optionally isolating and characterising the HCV-peptide from said complex,
- assaying said optionally isolated HCV-peptide or said complex in a T cell assay for T cell activation capacity and
- providing the optionally isolated HCV-peptide with a T cell activation capacity as HCV T cell epitope or as complex.

The method according to the present invention enables a screening system for screening binding capacity to specific MHC/HLA molecules. Identifying MHC binding molecules is an important tool for molecular characterisation of pathogens, tumors, etc. It is therefore possible with the present invention to screen a

variety (a "pool") of potential HCV-peptides as ligands at once for their functional affinity towards MHC molecules. Binding affinity towards MHC molecules is also a necessary prerequisite for HCV-peptides intended to be used as T cell epitopes, although not a sufficient one. Suitable HCV T cell epitope candidates have also to be screened and assayed with respect to their T cell activation capacity. The combination of the screening method for binding according to the present invention with a suitable T cell assay therefore provides the method for isolating HCV T cell epitopes according to the present invention wherein such T cell epitopes are identifiable out of a pool of potential HCV-peptides using an MHC binding assay.

In contrast to the prior art, where such assays have always been performed on ligands with known binding/MHC specificity, the methods according to the present invention provide such assays as a screening tool for pools with ligands of unknown specificity. In the prior art such assays have been typically performed on individual single ligands, to test their binding affinity to MHC/HLA molecules. In Kwok et al. (2001) pools of maximally up to 5 overlapping synthetic peptides were used to generate MHC class II tetramers; the latter were then used to stain PBMC for T cells specific for particular MHC class II:peptide complexes which were generated in the binding reaction with the pools of 5 peptides. However, an increase in the number of ligands per pool in such an approach was not regarded as being possible, both for sensitivity and specificity reasons (Novak et al. 2001). A problem with regard to specificity would be the generation of MHC tetramers with more than one binder per tetramer, if more than one binder would be present in the pool. This would preclude staining of T cells, which is used for identification of epitopes in the approach described in the prior art. In strong contrast to that the approach according to the present invention allows the identification of more than one binder out of highly complex mixtures containing more than one binder.

The nature of the pool to be screened with the present invention is not critical: the pools may contain any naturally or not naturally occurring HCV-peptide which a) binds specifically to

MHC/HLA molecules and/or b) may be specifically recognized by T cells. The binding properties of the set of HCV-peptides of the pool with respect to MHC molecules is not known; therefore, usually binders and at least a non-binder for a given MHC molecule are contained in the pool. The pool therefore comprises at least ten different HCV-peptides. Practically, pools are used according to the present invention containing significantly more different HCV-peptide species, e.g. 20 or more, 100 or more, 1.000 or more or 10.000 or more. It is also possible to screen larger libraries (with e.g. more than 10^6 , more than 10^8 or even more than 10^{10} different HCV-peptide species). This, however, is mainly dependent on the availability of such HCV-peptide libraries.

Preferred pools of ligands to be used in the method according to the present invention are selected from the group consisting of a pool of peptides, especially overlapping peptides, a pool of protein fragments, a pool of modified peptides, a pool obtained from antigen-presenting cells, preferably in the form of total lysates or fractions thereof, especially fractions eluted from the surface or the MHC/HLA molecules of these cells, a pool comprised of fragments of cells, especially HCV containing cells, tumor cells or tissues, especially from liver, a pool comprised of peptide libraries, pools of (poly)-peptides generated from recombinant DNA libraries, especially derived from pathogens or (liver) tumor cells, a pool of proteins and/or protein fragments from HCV or mixtures thereof.

The HCV-peptides of the pools may be derived from natural sources (in native and/or derivatised form) but also be produced synthetically (e.g. by chemical synthesis or by recombinant technology). If (poly)peptide ligands are provided in the pools, those peptides are preferably generated by peptide synthesizers or by recombinant technology. According to a preferred embodiment, a pool of (poly)peptides may be generated from recombinant DNA libraries, e.g. derived from HCV or HCV containing (tumor) cells, by in vitro translation (e.g. by ribosome display) or by expression through heterologous hosts like E.coli or others.

The nature of the specific MHC molecules (of course also MHC-

like molecules are encompassed by this term) to be selected for the present methods is again not critical. Therefore, these molecules may be selected in principle from any species, especially primates like humans (HLA, see below), chimpanzees, other mammals, e.g. macaques, rabbits, cats, dogs or rodents like mice, rats, guinea pigs and others, agriculturally important animals like cattle, horses, sheep and fish, although human (or "humanized") molecules are of course preferred for providing vaccines for humans. For providing vaccines for specific animals, especially agriculturally important animals, like cattle, horses, sheep and fish, the use of MHC molecules being specific for these animals is preferred.

Preferred HLA molecules therefore comprise Class I molecules derived from the HLA-A, -B or -C loci, especially A1, A2, A3, A24, A11, A23, A29, A30, A68; B7, B8, B15, B16, B27, B35, B40, B44, B46, B51, B52, B53; Cw3, Cw4, Cw6, Cw7; Class II molecules derived from the HLA-DP, -DQ or -DR loci, especially DR1, DR2, DR3, DR4, DR7, DR8, DR9, DR11, DR12, DR13, DR51, DR52, DR53; DP2, DP3, DP4; DQ1, DQ3, DQ5, DQ6; and non-classical MHC/HLA and MHC/HLA-like molecules, which can specifically bind ligands, especially HLA-E, HLA-G, MICA, MICB, Qa1, Qa2, T10, T18, T22, M3 and members of the CD1 family.

According to a preferred embodiment, the methods according to the present invention is characterised in that said MHC/HLA molecules are selected from HLA class I molecules, HLA class II molecules, non classical MHC/HLA and MHC/HLA-like molecules or mixtures thereof, or mixtures thereof.

Preferably, the optional characterising step of the HCV-peptides of the complex is performed by using a method selected from the group consisting of mass spectroscopy, polypeptide sequencing, binding assays, especially SDS-stability assays, identification of ligands by determination of their retention factors by chromatography, especially HPLC, or other spectroscopic techniques, especially violet (UV), infra-red (IR), nuclear magnetic resonance (NMR), circular dichroism (CD) or electron spin resonance (ESR), or combinations thereof.

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According to a preferred embodiment the method of the present invention is characterised in that it is combined with a cytokine secretion assay, preferably with an Elispot assay, an intracellular cytokine staining, FACS or an ELISA (enzyme-linked immunoassays) (see e.g. Current Protocols in Immunology).

Preferred T cell assays comprise the mixing and incubation of said complex with isolated T cells and subsequent measuring cytokine secretion or proliferation of said isolated T cells and/or the measuring up-regulation of activation markers, especially CD69, CD38, or down-regulation of surface markers, especially CD3, CD8 or TCR and/or the measuring up-/down-regulation of mRNAs involved in T cell activation, especially by real-time RT-PCR (see e.g. Current Protocols in Immunology, Current Protocols in Molecular Biology).

Further preferred T cell assays are selected from T cell assays measuring phosphorylation/de-phosphorylation of components downstream of the T cell receptor, especially p56 lck, ITAMS of the TCR and the zeta chain, ZAP70, LAT, SLP-76, fyn, and lyn, T cell assays measuring intracellular Ca^{++} concentration or activation of Ca^{++} -dependent proteins, T cell assays measuring formation of immunological synapses, T cell assays measuring release of effector molecules, especially perforin, granzymes or granulolysin or combinations of such T cell assays (see e.g. Current Protocols in Immunology, Current Protocols in Cell Biology).

In order to identify the molecular determinants of immune-protection against HCV a specific method of epitope capturing was applied using synthetic peptides representing the conserved parts of HCV genotypes 1, 2 and 3. Focusing on conserved regions ensures broad applicability of the epitopes. Moreover, these regions probably cannot easily be mutated by the virus, thus minimizing the danger of evolution of immune-escape variants.

With the methods of the present invention novel HCV-epitopes are detected. According to a further aspect, the present invention therefore also provides HCV T cell epitopes identifiable by a method according to the present invention, said T cell epitopes preferably being selected from the group consisting of poly-

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peptides comprising the peptides A120-A124, B25-B30, B46-B48, B84-B92, C106, C113-C114, 1627, 1628, 1629, 1604 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101, *0401, *0404, *0701 and thus covering at least 45-55% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides 1630, C97, 1547, B94-B98, A272-A276 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101, *0401, *0701 and thus covering at least 40-50% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides B120, B122, C108, C134, C152 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101, *0404, *0701 and thus covering at least 45% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides 1606, 1607, 1577, 1578 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0401, *0404, *0701 and thus covering at least 45% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides B50-52, 1623, C130 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101, *0401, *0404 and thus covering at least 40% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides 1603, C96 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101, *0701 and thus covering at least 40% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides C191 according to Table 1, being a novel ligand for at least HLA-DRB1*0401, *0701 and thus covering at least 40% of major populations (see Tab. 2).

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Preferred polypeptides are selected from the group comprising the peptides A216-A224, A242-A244, C92-C93 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101, *0401 and thus covering at least 35% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptide A174 according to Table 1 or 2, being a novel ligand for at least HLA-DRB1*0404, *0701 and thus covering at least 25-30% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides B32-B38, B100-B102, C135 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101, *0404 and thus covering at least 20-25% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptide C162 according to Table 1 or 2, being a novel ligand for at least HLA-DRB1*0401, *0404 and thus covering at least 20-25% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides 1618, 1622, 1624, 1546, 1556 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0701 and thus covering at least 25% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides A114, B58, B112-B118, B18-B22, C112, C116, C122, C127, C144, C159-C160, C174, 1558, 1581 according to Table 1 or 2. These peptides are novel ligands for at least HLA-DRB1*0101 and thus covering at least 20% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptide C95, being a novel ligand for at least HLA-DRB1*0401 and thus covering at least 20% of major populations (see Tab. 2).

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Preferred polypeptides are selected from the group comprising the peptides C129, C157-C158, A254-A258, 1605, C109, C161 according to Table 1 or 2. These peptides comprising novel ligands for at least HLA-DRB1*0404 and thus covering at least 5% of major populations (see Tab. 2).

Preferred polypeptides are selected from the group comprising the peptides 1547, 1555, 1558, 1559, 1560, 1563, 1592, 1604, 1605, 1616, 1621, 1623, 1625, 1627, 1630, 1649, 1650, 1651, 1652, 1654, 1655, 1656 according to Table 1 or 2, these peptides displaying immunogenicity in HLA-DRB1*0401 transgenic mice (see Example II) and thus representing or containing a confirmed HLA class II T-cell epitope binding to at least HLA-DRB1*0401 (see Tab. 3).

Preferred polypeptides are selected from the group comprising the peptides 1545, 1552, 1555, 1558, 1559, 1560, 1577, 1592, 1604, 1605, 1615, 1617, 1621, 1627, 1631, 1632, 1641, 1647, 1650, 1651, 1652, 1653, 1654, 1655 according to Table 1 or 2, these peptides displaying immunogenicity in HLA-A*0201 transgenic mice (see Example II) and thus representing or containing a confirmed HLA class I T-cell epitope binding to at least HLA-A*0201 (see Tab. 3).

Preferred polypeptides which are shown to be HLA-B*0702 epitopes with T-cell activating capacity are selected from the group consisting of polypeptides 1506, 1526, 1547, 1552, 1553, 1555, 1558, 1562, 1563, 1565, 1577, 1578, 1580, 1587, 1592, 1604, 1605, 1621, 1623, 1624, 1627, 1628, 1647, 1650, 1651, 1843 with sequence LPRRGPR (contained in 1506) and 1838 with sequence SP-GALVGV (contained in 1587) as minimal HLA-B*0702 epitopes.

Peptides 1526, 1565, 1631 are also shown to be immunogenic in HLA-DRB1*0401 transgenic mice contain known class II epitopes. Peptides 1526, 1553, 1565, 1587, 1623, 1630 are also shown to be immunogenic in HLA-A*0201 transgenic mice contain known A2 epitopes.

Preferred polypeptides are selected from the group comprising

the peptides listed in tables 3, 5 and the bold peptides in 7 ("hotspots").

The preferred polypeptides mentioned above also include all fragments containing the minimal sequence of the epitope, i.e. the 8- or 9-mer being necessary for binding to MHC/HLA molecules.

Preferably, the epitopes or peptides according to the present invention further comprises 1 to 30, preferably 2 to 10, especially 2 to 6, naturally occurring amino acid residues at the N-terminus, the C-terminus or at the N- and C-terminus. For the purposes of the present invention the term "naturally occurring" amino acid residue relates to amino acid residues present in the naturally occurring protein at the specific position, relative to the epitope or peptide. For example, for the HLA-A2 epitope with the amino acid sequence HMWNFISGI contained within peptide ID 1565 (Tab. 1), the naturally occurring amino acid residue at the N-terminus is -K; the three naturally occurring amino acid residues at the C-terminus are -QYL. A "non-naturally occurring" amino acid residue is therefore any amino acid residue being different as the amino acid residue at the specific position relative to the epitope or peptide.

According to a preferred embodiment of the present invention, the present epitopes or peptides further comprise non-naturally occurring amino acid(s), preferably 1 to 1000, more preferred 2 to 100, especially 2 to 20 non-naturally occurring amino acid residues, especially at the N-terminus, the C-terminus or at the N- and C-terminus. Also combinations of non-naturally and naturally occurring amino acid residues are possible under this specific preferred embodiment. The present epitope may also contain modified amino acids (i.e. amino acid residues being different from the 20 "classical" amino acids, such as D-amino acids or S-S bindings of Cys) as additional amino acid residues or in replacement of a naturally occurring amino acid residue.

It is clear that also epitopes or peptides derived from the present epitopes or peptides by amino acid exchanges improving, conserving or at least not significantly impeding the T cell ac-

tivating capability of the epitopes are covered by the epitopes or peptides according to the present invention. Therefore, the present epitopes or peptides also cover epitopes or peptides, which do not contain the original sequence as derived from a specific strain of HCV, but trigger the same or preferably an improved T cell response. These epitopes are referred to as "heteroclitic". These include any epitope, which can trigger the same T cells as the original epitope and has preferably a more potent activation capacity of T cells preferably in vivo or also in vitro. Also the respective homologous epitopes from other strains of HCV are encompassed by the present invention.

Heteroclitic epitopes can be obtained by rational design i.e. taking into account the contribution of individual residues to binding to MHC/HLA as for instance described by Ramensee et al. 1999 or Sturniolo et al. 1999, combined with a systematic exchange of residues potentially interacting with the TCR and testing the resulting sequences with T cells directed against the original epitope. Such a design is possible for a skilled man in the art without much experimentation.

Another possibility includes the screening of peptide libraries with T cells directed against the original epitope. A preferred way is the positional scanning of synthetic peptide libraries. Such approaches have been described in detail for instance by Blake et al 1996 and Hemmer et al. 1999 and the references given therein.

As an alternative to epitopes represented by the cognate HCV derived amino acid sequence or heteroclitic epitopes, also substances mimicking these epitopes e.g. "peptidemimetica" or "retro-inverso-peptides" can be applied.

Another aspect of the design of improved epitopes is their formulation or modification with substances increasing their capacity to stimulate T cells. These include T helper cell epitopes, lipids or liposomes or preferred modifications as described in WO 01/78767.

Another way to increase the T cell stimulating capacity of epi-

topes is their formulation with immune stimulating substances for instance cytokines or chemokines like interleukin-2, -7, -12, -18, class I and II interferons (IFN), especially IFN-gamma, GM-CSF, TNF-alpha, flt3-ligand and others.

According to a further aspect, the present invention is drawn to the use of a HCV epitope or HCV peptide according to the present invention for the preparation of a HLA restricted vaccine for treating or preventing hepatitis C virus (HCV) infections.

The invention also encompasses the use of an epitope according to the present invention for the preparation of a vaccine for treating or preventing hepatitis C virus (HCV) infections.

Consequently, the present invention also encompasses a vaccine for treating or preventing hepatitis C virus (HCV) infections comprising an epitope according to the present invention.

Furthermore, also a HLA specific vaccine for treating or preventing hepatitis C virus (HCV) infections comprising the epitopes or peptides according to the present invention is an aspect of the present invention.

Preferably, such a vaccine further comprises an immunomodulating substance, preferably selected from the group consisting of polycationic substances, especially polycationic polypeptides, immunomodulating nucleic acids, especially deoxyinosine and/or deoxyuracile containing oligodeoxynucleotides, or mixtures thereof.

Preferably the vaccine further comprises a polycationic polymer, preferably a polycationic peptide, especially polyarginine, polylysine or an antimicrobial peptide.

The polycationic compound(s) to be used according to the present invention may be any polycationic compound, which shows the characteristic effect according to the WO 97/30721. Preferred polycationic compounds are selected from basic polypeptides, organic polycations, basic polyaminoacids or mixtures thereof.

These polyaminoacids should have a chain length of at least 4 amino acid residues. Especially preferred are substances containing peptidic bounds, like polylysine, polyarginine and polypeptides containing more than 20%, especially more than 50% of basic amino acids in a range of more than 8, especially more than 20, amino acid residues or mixtures thereof. Other preferred polycations and their pharmaceutical compositions are described in WO 97/30721 (e.g. polyethyleneimine) and WO 99/38528. Preferably these polypeptides contain between 20 and 500 amino acid residues, especially between 30 and 200 residues.

These polycationic compounds may be produced chemically or recombinantly or may be derived from natural sources.

Cationic (poly)peptides may also be polycationic anti-bacterial microbial peptides. These (poly)peptides may be of prokaryotic or animal or plant origin or may be produced chemically or recombinantly. Peptides may also belong to the class of defensins. Such host defense peptides or defensins are also a preferred form of the polycationic polymer according to the present invention. Generally, a compound allowing as an end product activation (or down-regulation) of the adaptive immune system, preferably mediated by APCs (including dendritic cells) is used as polycationic polymer.

Especially preferred for use as polycationic substance in the present invention are cathelicidin derived antimicrobial peptides or derivatives thereof (WO 02/13857), incorporated herein by reference), especially antimicrobial peptides derived from mammal cathelicidin, preferably from human, bovine or mouse, or neuroactive compounds, such as (human) growth hormone (as described e.g. in WO01/24822).

Polycationic compounds derived from natural sources include HIV-REV or HIV-TAT (derived cationic peptides, antennapedia peptides, chitosan or other derivatives of chitin) or other peptides derived from these peptides or proteins by biochemical or recombinant production. Other preferred polycationic compounds are cathelin or related or derived substances from cathelin, especially mouse, bovine or especially human cathelins and/or

cathelicidins. Related or derived cathelin substances contain the whole or parts of the cathelin sequence with at least 15-20 amino acid residues. Derivations may include the substitution or modification of the natural amino acids by amino acids, which are not among the 20 standard amino acids. Moreover, further cationic residues may be introduced into such cathelin molecules. These cathelin molecules are preferred to be combined with the antigen/vaccine composition according to the present invention. However, these cathelin molecules surprisingly have turned out to be also effective as an adjuvant for a antigen without the addition of further adjuvants. It is therefore possible to use such cathelin molecules as efficient adjuvants in vaccine formulations with or without further immunactivating substances.

Another preferred polycationic substance to be used according to the present invention is a synthetic peptide containing at least 2 KLK-motifs separated by a linker of 3 to 7 hydrophobic amino acids, especially L (WO 02/32451, incorporated herein by reference).

The immunomodulating (or:immunogenic) nucleic acids to be used according to the present invention can be of synthetic, prokaryotic and eukaryotic origin. In the case of eukaryotic origin, DNA should be derived from, based on the phylogenetic tree, less developed species (e.g. insects, but also others). In a preferred embodiment of the invention the immunogenic oligodeoxynucleotide (ODN) is a synthetically produced DNA-molecule or mixtures of such molecules. Derivatives or modifications of ODNs such as thiophosphate substituted analogues (thiophosphate residues substitute for phosphate) as for example described in US patents US 5,723,335 and US 5,663,153, and other derivatives and modifications, which preferably stabilize the immunostimulatory composition(s) but do not change their immunological properties, are also included. A preferred sequence motif is a six base DNA motif containing an (unmethylated) CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (5'-Pur-Pur-C-G-Pyr-Pyr-3'). The CpG motifs contained in the ODNs according to the present invention are more common in microbial than higher vertebrate DNA and display differences in the pattern of methyl-

ation. Surprisingly, sequences stimulating mouse APCs are not very efficient for human cells. Preferred palindromic or non-palindromic ODNs to be used according to the present invention are disclosed e.g. in Austrian Patent applications A 1973/2000, A 805/2001, EP 0 468 520 A2, WO 96/02555, WO 98/16247, WO 98/18810, WO 98/37919, WO 98/40100, WO 98/52581, WO 98/52962, WO 99/51259 and WO 99/56755 all incorporated herein by reference. Apart from stimulating the immune system certain ODNs are neutralizing some immune responses. These sequences are also included in the current invention, for example for applications for the treatment of autoimmune diseases. ODNs/DNAs may be produced chemically or recombinantly or may be derived from natural sources. Preferred natural sources are insects.

Alternatively, also nucleic acids based on inosine and cytidine (as e.g. described in the WO 01/93903) or deoxynucleic acids containing deoxy-inosine and/or deoxyuridine residues (described in WO 01/93905 and PCT/EP 02/05448; incorporated herein by reference) may preferably be used as immunostimulatory nucleic acids for the present invention.

Of course, also mixtures of different immunogenic nucleic acids may be used according to the present invention.

Preferably, the present vaccine further comprises a pharmaceutically acceptable carrier.

According to a further preferred embodiment, the present vaccine comprises an epitope or peptide which is provided in a form selected from peptides, peptide analogues, proteins, naked DNA, RNA, viral vectors, virus-like particles, recombinant/chimeric viruses, recombinant bacteria or dendritic cells pulsed with protein/peptide/RNA or transfected with DNA comprising the epitopes or peptides.

According to a further aspect, the present invention is drawn to T cells, a T cell clone or a population (preparation) of T cells specifically recognizing any HCV epitope or peptide according to the present invention, especially a HCV epitope as described above. A preferred application of such T cells is their expan-

sion in vitro and use for therapy of patients e.g. by adoptive transfer. Therefore, the present invention also provides the use of T cells, a T cell clone or a population (preparation) of T cells for the preparation of a composition for the therapy of HCV patients.

Such T cells (clones or lines) according to the present invention, specifically those recognizing the aforementioned HCV peptides are also useful for identification of heteroclitic epitopes, which are distinct from the originally identified epitopes but trigger the same T cells.

Such cells, compositions or vaccines according to the present invention are administered to the individuals in an effective amount.

According to a further aspect, the present invention also relates to the use of the peptides with formulae QRKTKRNTN, QRKTKRNT, or 1615, 1616, 1617 in particular 9meric peptides derived from the latter 3 peptides with formulae SAKSKFGYG, SAKSKYGYG, or SARSKYGYG as HLA-B*08 epitopes, especially for the preparation of a pharmaceutical preparation for a HLA-B*08 specific vaccine; the use of the peptides with the formulae RKTKRNTNR as HLA-B*2705 epitope, especially for the preparation of a pharmaceutical preparation for a HLA-B*2705 specific vaccine; and the use of the peptides with the formulae ARLIVFPDL as HLA-B*2705 and HLA-B*2709 specific vaccine. Further, it also relates to the use of the hotspot epitopes selected from the group of peptides 1835, 84EX, 87EX, 89EX, 1426, 1650, 1836, 1846, 1651, 1800, 1799, C114, 1827, C112, C114EX, 1827EX, 1798, 1604, 1829, 1579, 1624, 1848, 1547, A1A7, A122EX, A122, 1825, A241, B8B38, C70EX, C92, C97, C106, and C134 according to table 7 for the preparation of a vaccine comprising synthetic peptides, recombinant protein and/or DNA constitutes of such epitopes.

In particular, two or more epitope hotspots can be combined, with or without linker sequences. Preferred linker sequences consist for instance of 3 to 5 glycine, or alanine or lysine residues. This may be achieved by peptide synthesis. However, combination of hotspots may result in quite long polypeptides.

In this case, cloning DNA encoding for such constructs and expressing and purifying the corresponding recombinant protein is an alternative. Such recombinant proteins can be used as antigens, which in combination with the right adjuvant (IC31, pR,...) can elicit T-cell responses against all the epitopes they harbor. At the same time, such artificial polypeptides are devoid of the activities (enzymatic, toxic, immuno-suppressive, ...), the natural HCV antigens may possess.

There are several other ways of delivering T-cell epitope hot-spots or combinations thereof. These include: recombinant viral vectors like vaccinia virus, canary pox virus, adenovirus; self-replicating RNA vectors; "naked DNA" vaccination with plasmids encoding the hotspots or combination thereof; recombinant bacteria (e.g. Salmonella); dendritic cells pulsed with synthetic peptides, or recombinant protein, or RNA or transfected with DNA, each encoding T-cell epitope hotspots or combinations thereof.

The invention will be explained in more detail by way of the following examples and drawing figures, to which, however it is not limited.

Fig.1 shows 40 peptide mixtures each containing up to 20 HCV derived 15- to 23mer peptides.

Fig.2 shows the Epitope Capture approach using peptide pools and empty DRB1*0401 molecules.

Fig.3 shows the Epitope Capture approach using peptide pools and empty DRB1*0404 molecules.

Fig.4 shows binding of individual peptides to DRB1*0401.

Fig.5 shows binding of individual peptides to DRB1*0404.

Fig.6 shows binding of individual peptides to DRB1*0101.

Fig.7 shows peptides binding to DRB1*0701.

Fig.8 shows mouse IFN-gamma ELISpot with splenocytes or separated CD8+ or CD4+ cells from HLA-DRB1*0401 tg mice vaccinated with Ipep1604+IC31.

Fig.9 shows mouse IFN-gamma ELISpot with splenocytes or separated CD8+ or CD4+ cells from HLA-A*0201 tg mice vaccinated with Ipep1604+IC31.

Fig.10 shows mouse IFN-gamma ELISpot with splenocytes or separated CD8+ or CD4+ cells from HLA-B*0702tg mice vaccinated with Ipep1604+IC31.

E x a m p l e s :

General description of the examples:

The present examples show the performance of the present invention on a specific pathogen hepatitis C virus (HCV).

In the first part the method according to the present invention was applied, which is based on the use of "empty HLA molecules". These molecules were incubated with mixtures of potential HCV derived peptide ligands, screening for specific binding events. The possibility to use highly complex mixtures allows a very quick identification of the few binders out of hundreds or even thousands of potential ligands. This is demonstrated by using HLA-DRB1*0101, -DRB1*0401, -DRB1*0404, -DRB1*0701 molecules and pools of overlapping 15- to 23mers. Importantly, this analysis using multiple different HLA-alleles allows identifying promiscuous ligands capable to binding to more than one HLA allele. Promiscuous T-cell epitopes are particularly valuable components of epitope-based vaccines. They enable treating a higher portion of a population than epitopes restricted to one HLA allele.

The same process can be applied for class I molecules and peptides of appropriate length i.e. 8 to 11-mers. The ligand-pools can be synthetic overlapping peptides. Another possibility is to digest the antigen in question enzymatically or non-enzymatically. The latter achieved by alkali-hydrolysis generates all po-

tential degradation products and has been successfully used to identify T cell epitopes (Gavin 1993). Enzymatic digestions can be done with proteases. One rational way would further be to use proteases involved in the natural antigen-processing pathway like the proteasome for class I restricted epitopes (Heemels 1995) or cathepsins for class II restricted epitopes (Villadangos 2000). Ligand pools could also be composed of naturally occurring ligands obtained for instance by lysis of or elution from cells carrying the respective epitope. In this regard it is important to note that also non-peptide ligands like for instance glycolipids can be applied. It is known that non-classical class I molecules, which can be encoded by the MHC (e.g. HLA-G, HLA-E, MICA, MICB) or outside the MHC (e.g. CD1 family) can present various non-peptide ligands to lymphocytes (Kronenberg 1999). Use of recombinant "empty" nonclassical class I molecules would allow binding reactions and identification of binders in similar manner as described here.

After rapid identification of ligands capable of binding to HLA molecules the process according to the present invention also offers ways to characterize directly specific T cell responses against these binders. One possibility is to directly use the isolated HLA:ligand complex in a so called "synthetic T cell assay". The latter involves antigen-specific re-stimulation of T cells by the HLA:ligand complex together with a second signal providing co-stimulation like activation of CD28 by an activating antibody. This assay can be done in an ELISpot readout.

Another possibility is the immunization of HLA-transgenic mice to prove immunogenicity of ligands identified by the Epitope Capture approach as demonstrated in Example II.

MATERIALS & METHODS

Peptides

In order to identify conserved regions between HCV genotypes 1, 2 and 3, about 90 full genomes publicly available through Genbank were aligned. In total, 43% of the coding region of HCV was found to be conserved in at least 80% of clinical isolates. In

cases, where at a certain position consistently two distinct amino acids (eg. arginine or lysine) were found, both variants were considered for analysis. Altogether 148 conserved regions, longer than 8 amino acids were identified. Conserved region were spanned by ~500 fifteen amino acid residue (15mer) peptides, each peptide overlapping its precursor by 14 out of 15 amino acids. Conserved regions between 8 and 14 amino acids long were covered by further 80 (non-overlapping) 15mers. 15mers were synthesized using standard F-moc chemistry in parallel (288 at a time) on a Syro II synthesizer (MultisynTech, Witten, Germany). Each fourth 15mer was checked by mass spectrometry. 15mers were applied for experiments without further purification. In addition 63 peptides of 16-xx aa were synthesized using standard F-moc chemistry on an ABI 433A synthesizer (Applied Biosystems, Weiterstadt, Germany) and purified by RP-HPLC (Biocut 700E, Applied Biosystems, Langen, Germany) using a C18 column (either ODS ACU from YMC or 218TP, Vydac). Purity and identity were characterized by MALDI-TOF on a Reflex III mass-spectrometer (Bruker, Bremen, Germany). Peptides were solubilized in 100 % DMSO at ~10 mg/ml (~5 mM). Stocks of peptide pools (20 peptides each) were made in 100 % DMSO at a final concentration of 0.5 mg/ml (~0.25 mM) for each peptide. All peptides used in the present invention are listed in Table 1. Peptides YAR (YARFQSQTTLKQKT), HA (PKYVKQNTLKLAT), P1 (GYKVLVLNPSVAAT), P2 (HMWNFISGIQYLSTLPGNPA), P3 (KFPGGGQIVGVYLLPRRRGPRL), P4 (DLMGYIPAV) and CLIP (KLPKPPKPVSKMRMATPLLMQALPM) were used as control peptides in binding assays.

Epitope capture and peptide binding assay

Soluble HLA class II DRA1*0101/DRB1*0101/Ii, DRA1*0101/DRB1*0401/Ii, DRA1*0101/DRB1*0404/Ii and DRA1*0101/DRB1*0701/Ii molecules were expressed in SC-2 cells and purified as described in Aichinger et al., 1997. In peptide binding reactions soluble DRB1*0101, DRB1*0401, DRB1*0404 molecules were used in a concentration of ~0.5 µM, and each single peptide was added in 10-fold molar excess (5 µM) if not mentioned differently. The concentration of DMSO in the binding reaction did not exceed 4 %. The reaction was performed in PBS buffer (pH 7.4) at room temperature for 48 hours in the presence

of a protease inhibitor cocktail (Roche) and 0.1 % octyl-beta-D-glucopyranoside (Sigma). Peptide binding was evaluated in an SDS-stability assay (Gorga et al., 1987): trimeric HLA class II alpha:beta:peptide complexes are resistant to SDS and consequently appear as ~60 kDa band in SDS-PAGE Western blot analysis. Individual HLA class II alpha- and beta-chains not stabilized by bound peptide migrate as ~35 kDa and ~25 kDa bands, respectively. Briefly, HLA-peptide complexes were treated with 1 % SDS at room temperature and resolved by SDS-PAGE run with 20 mA for approximately 2.5 hours at room temperature. Protein was transferred onto PVDF membrane by electroblotting, and stained with anti-alpha-chain TAL.1B5 or/and beta-chain MEM136 antibodies. For detection of Western-blot signals ECL solutions (Amersham) were used. For DRB1*0101 molecules HA and P1 peptides were used as controls for evaluation of strong binding, P2 peptide for intermediate binding and YAR as a negative control. For DRB1*0401 the strongest binding controls were YAR and HA peptides, while P1 and P2 served as an intermediate and weak binder, respectively. In the case of DRB1*0404 molecules P1 and P2 peptides were used to estimate strong binding, YAR peptide to control intermediate binding and HA peptide as a negative control. The binding affinities to DRB1*0701 were test by a peptide-competition assay (Reay et al., 1992). Briefly, binding of the biotinylated CLIP peptide with high affinity (reference peptide) has been used for monitoring of HLA:peptide complex formation. A testing peptide added to the binding reaction at an equimolar concentration to CLIP peptide could compete out CLIP when its affinity is higher or inhibit binding for 50 % if its affinity is equal to affinity of CLIP. In the case of lower affinity peptides they should be added in excess to the reference peptide to compete for occupancy of HLA binding groove. The values of the concentration of competitor peptides required for 50 % inhibition of reference peptide (biotinylated CLIP) binding (IC_{50}) can be used for evaluation of peptide binding affinities. Alternatively, comparing of the amount of reference peptide bound to HLA molecules in the presence or absence of competitor peptide one can determine the binding activity of the peptide of interest. In the present peptide-competition assay conditions of peptide binding were similar to described above. DRB1*0701 molecules were used in a concentration of ~0.5 μ M and biotinylated

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CLIP was added to all samples in the final concentration of 2 μ M. Competitor peptides were added in three different concentrations: 2 nM, 20 μ M and 200 μ M. Binding reaction was performed in PBS buffer (pH 7.4) for 18 hours at 37°C. The amount of biotinylated CLIP associated with soluble DRB1*0701 molecules was determined by ELISA. Briefly, MaxiSorp 96-well plates (Nunc, Denmark) were coated with mouse anti-DR antibody L243 by overnight incubation with 50 μ l of 10 μ g/ml dilution in PBS at 4°C. Non-specific binding to wells was blocked by incubation with T-PBS containing 3 % of BSA for 2 hours at 37°C and binding reactions were then "captured" for 2 hours at room temperature. Following extensive washing, HLA-associated peptide complexes were detected using alkaline phosphatase-streptavidin (Dako) and Sigma 104 phosphatase substrate. A microplate reader (VICTOR) was used to monitor optical density at 405 nm. Non-biotinylated CLIP, P1 and P2 peptides were used as positive controls to evaluate strong binding. Peptide P3 and P4 served as a weakly binding and non-binding control, respectively.

Immunization of HLA-transgenic mice

Immunogenicity of synthetic HCV-derived peptides was tested in HLA-DRB1*0401- and HLA-A*0201-transgenic mice as follows: Groups of 3 mice (female, 8 weeks of age) were injected subcutaneously into the flank (in total 100 μ g of peptide + 30 μ g oligodeoxynucleotide CpI (Purimex, Göttingen, Germany) per mouse). One week after the vaccination, spleens were removed and the splenocytes were activated ex vivo with the peptide used for vaccination and an irrelevant negative control peptide to determine IFN-gamma-producing specific cells (mouse ELISpot assay).

Mouse splenocyte ELISpot assay for single cell IFN-gamma release ELISpot plates (MAHA S4510, Millipore, Germany) were rinsed with PBS (200 μ l/well), coated with anti-mouse IFN-gamma mAb (clone R46A2; 100 μ l/well of 5 μ g/ml in 0.1 M NaHCO₃, pH 9.2-9.5) and incubated overnight at 4°C. Plates were washed four times with PBS/0.1% Tween 20 and incubated with PBS/1% BSA (200 μ l/well) at room temperature for 2 h to block nonspecific binding. Spleen cells from vaccinated mice were prepared and plated at 1×10^6 - 3×10^5 cells/well and incubated overnight at 37°C/5% CO₂ either

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in the presence of the immunizing antigen (peptide), control peptides or with medium alone. Subsequently, plates were washed four times and incubated with biotinylated anti-mouse IFN-gamma mAb (clone AN18.17.24, 100 µl/well of 2 µg/ml in PBS/1% BSA) for 2 h at 37°C. After washing, streptavidin-peroxidase (Roche Diagnostics, Vienna, Austria) was added (1/5000 in PBS, 100 µl/well) and plates were incubated at room temperature for 2 additional hours. Subsequently, substrate was added to the washed plates (100 µl/well of a mixture of 10 ml 100 mM Tris pH 7.5 supplemented with 200 µl of 40 mg/ml DAB stock containing 50 µl of 80 mg/ml NiCl₂ stock and 5 µl of 30% H₂O₂). The reaction was stopped after 20-30 minutes by washing the plates with tap water. Dried plates were evaluated with an ELISpot reader (BIOREADER 2000, BioSys, Karben, Germany).

IFN-gamma ELISpot with human PBMC

PBMC from HCV RNA-negative therapy responders or subjects spontaneously recovered were collected and HLA-typed serologically. Whole blood was collected in ACD Vacutainer tubes (Becton Dickinson Europe, Erembodegem, Germany). PBMC were isolated on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) using Leuco-sep tubes (Greiner, Frickenhausen, Germany), washed 3x with PBS (Invitrogen Life Technologies (formerly GIBCOBRL), Carlsbad, CA, USA) and resuspended at a concentration of 2×10^7 /ml in freezing medium consisting of 4 parts RPMI 1640 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 µM 2-mercaptoethanol (all from Invitrogen Life Technologies), 9 parts foetal bovine serum (FCS; from PAA, Linz, Austria) and 1 part DMSO (SIGMA, Deisenhofen, Germany). PBMC were stored over night in 1°C freezing containers (Nalgene Nunc International, Rochester, New York, USA) at -80°C and then transferred into liquid nitrogen. The ELISpot assay was essentially done as described (Lalvani et al.). Briefly, Multi Screen 96-well filtration plates MAIP S4510 (Millipore, Bedford, MA) were coated with 10 µg/ml (0,75 µg/well) anti-human IFN-g monoclonal antibody (Mab) B140 (Bender Med Systems, Vienna, Austria) over night at 4°C. Plates were washed 2 times with PBS (Invitrogen Life Technologies) and blocked with ELISpot medium (RPMI 1640 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 µM 2-mercaptoethanol (all from In-

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vitrogen Life Technologies) and 10% human serum type AB (PAA, Linz, Austria). Cryo-preserved PBMC were thawed quickly in a 37°C water bath, washed 1x with ELISPOT medium and incubated overnight (37°C, 5% CO₂). The next day cells were plated at 200,000 PBMC/well and co-cultivated with either individual peptides (10 µg/ml) or peptide pools (each peptide at a final concentration of 5 µg/ml) for 20 hrs. After removing cells and washing 6 times with wash buffer (PBS; 0,1% Tween 20 from SIGMA), 100 µl of a 1:10000 dilution (0.015 µg/well) of the biotinylated anti-human IFN-γ MAb B308-BT2 (Bender Med Systems), was added for an incubation of 2 hrs at 37°C or alternatively for over night at 4°C. After washing, Streptavidin-alkaline phosphatase (DAKO, Glostrup, Denmark) was added at 1.2 µg/ml for 1 hr at 37°C. The assay was developed by addition of 100 µl/well BCIP/NBT alkaline phosphatase substrate (SIGMA).

In vitro priming of human PBMCs

Human PBMCs are repeatedly stimulated with antigen (peptide or peptide mixture) in the presence of IL-2 and IL-7. This leads to the selective oligoclonal expansion of antigen-specific T cells. Responses against individual epitopes can be assessed for instance by IFN-γ ELISPOT assays. Freshly thawed PBMCs were cultured in 6 well plates (2-4x 10⁶/mL viable cells) in RPMI-1640 (GibcoBRL), 1% non-essential amino acids (GibcoBRL, cat# 11140-035), 1% Penicillin (10,000 U/ml)-Streptomycin (10,000 µg/ml) (GibcoBRL, cat#15140-122), 1% L-Glutamine (GibcoBRL), 0.1% beta-mercapto-ethanol (GibcoBRL), 1% Na-pyruvate (GibcoBRL), plus 10% Human AB serum (PAA, Linz, Austria). Peptides (10µM each) were added to each well. rhIL-7 (Strathmann Biotech) was added at 10 ng/mL final concentration. 20-30 U/mL rhIL-2 (Strathmann Biotech) were added on day 4. On day 10, all cells were removed from plates, washed once in media (as above), and counted. For the next cycle of in vitro priming, viable cells were co-cultivated with autologous gamma irradiated (1.2 gray/min, for 20 minutes) PBMC as feeders (plated at 100,000 per well) and peptides, rh-IL-2 as described above. ELISPOT was done as described above, except that 200,000 responder cells (pre-stimulated for 2 rounds of in vitro priming) were used together with 60,000 autologous irradiated responder cells.

Example I. Rapid identification of promiscuous HLA-binding peptides from HCV by measuring peptide pools arrayed in matrix format

To span conserved regions within the HCV polyprotein more than 640 peptides were synthesized (Table 1). For rapid identification of HLA ligands and novel T-cell epitopes, 40 peptide pools each containing 20 single peptides were prepared. The pools were constructed in a way that each peptide was present in 2 pools (matrix format). This allows identification of reactive peptides at the crossover points of row- and column mixtures (Fig. 1 HCV peptide matrix).

Table 1. Synthetic peptides derived from conserved regions of HCV.

Peptide ID	Peptide	Peptide ID	Peptide	Peptide ID	Peptide	Peptide ID	Peptide
A 1	MTSNPKPQRKTKRNT	A 79	HRSRNVGKVIDTLTC	A 197	CTVNYTIFKIRMYVG	A 239	ADGGCSGGAYDIIIC
A 2	STNPKPQRKTKRNTN	A 80	RSRNVGKVIDTLTCG	A 198	TVNYTIFKIRMYVGG	A 240	DGGCSGGAYDIIICD
A 3	TNPKPQRKTKRNTNR	A 81	SRNVGKVIDTLTCGF	A 199	VNYTIFKIRMYVGGV	A 241	GGCSGGAYDIIICDE
A 4	NPKPQRKTKRNTNRR	A 82	RNVGKVIDTLTCGFA	A 200	NYTIFKIRMYVGGVE	A 242	GCSGGAYDIIICDEC
A 5	PKPQRKTKRNTNRRP	A 83	NVGKVIDTLTCGFAD	A 201	YTFKIRMYVGGVEH	A 243	CSGGAYDIIICDECH
A 6	KPQRKTKRNTNRRPQ	A 84	VGKVIDTLTCGFADL	A 202	TIFKIRMYVGGVEHR	A 244	SGGAYDIIICDECHS
A 7	PQRKTKRNTNRRPQD	A 85	TLTCGFADLMGYIPV	A 203	IFKIRMYVGGVEHRL	A 245	TLIGIGTVLDOAET
A 8	QRKTKRNTNRRPQDV	A 86	LCGFADLMGYIPVV	A 204	PLALSTGLIHLHQN	A 246	TILGIGTVLDOAETA
A 9	RKTKRNTNRRPQDVK	A 87	TCGFADLMGYIPVVG	A 205	PALSTGLIHLHQNIV	A 247	ILGIGTVLDOAETAG
A 10	KTAKRNTNRRPQDVKF	A 88	CGFADLMGYIPVGA	A 206	ALSTGLIHLHQNIVD	A 248	LGIGTVLDOAETAGA
A 11	TKRNTNRRPQDVKFP	A 89	GFADLMGYIPVVGAP	A 207	LSTGLIHLHQNIVDV	A 249	GIGTVLDOAETAGAR
A 12	KRNTNRRPQDVKFPG	A 90	FADLMGYIPVVGAPL	A 208	STGLIHLHQNIVDVQ	A 250	IGTVLDOAETAGARL
A 13	RNTNRRPQDVKFPGG	A 91	ADLMGYIPVVGAPLG	A 209	TGLIHLHQNIVDVQY	A 251	GTVLDOAETAGARLV
A 14	NTNRRPQDVKFPGGG	A 92	DLMGYIPVVGAPLGG	A 210	GLIHLHQNIVDVQYL	A 252	TVLDOAETAGARLVV
A 15	NRNRRPQDVKFPGGGQ	A 93	ARALAHGVRVLEDGV	A 211	LHLHQNIVDVQYLY	A 253	VLDOAETAGARLVVL
A 16	NRRPQDVKFPGGGQI	A 94	RALAHGVRVLEDGVN	A 212	LHLHQNIVDVQYLYG	A 254	LDQAETAGARLVVLA
A 17	RRPQDVKFPGGGQIV	A 95	ALAHGVRVLEDGVNY	A 213	LPALSTGLIHLHQN	A 255	QAETAGARLVVLAT
A 18	RPQDVKFPGGGQIVG	A 96	LAHGVRVLEDGVNYA	A 214	PALSTGLIHLHQNIV	A 256	QAETAGARLVVLATA
A 19	PQDVKFPGGGQIVGG	A 97	AHGVRVLEDGVNYAT	A 215	ALSTGLIHLHQNIVD	A 257	QAETAGARLVVLATAT
A 20	QDVKFPGGGQIVGGV	A 98	HGVRVLEDGVNYATG	A 216	LSTGLIHLHQNIVDVQ	A 258	ETAGARLVVLATATP
A 21	DKFPGGGQIVGGVY	A 99	GVRVLEDGVNYATGN	A 217	STGLIHLHQNIVDVQ	A 259	TAGARLVVLATATPP
A 22	VKFPGGGQIVGGVYL	A 100	VRVLEDGVNYATGNL	A 218	TGLIHLHQNIVDVQY	A 260	AGARLVVLATATPPG
A 23	KFPGGGQIVGGVYLL	A 101	RVLEDGVNYATGNLP	A 219	GLIHLHQNIVDVQYMY	A 261	GARLVVLATATPPGS
A 24	FPGGGQIVGGVYLLP	A 102	VLEDGVNYATGNLPG	A 220	LHLHQNIVDVQYMYG	A 262	RLVVLATATPPGSV
A 25	PGGGQIVGGVYLLPR	A 103	LEDGVNYATGNLPGC	A 221	LHLHQNIVDVQYMYG	A 263	RVVLATATPPGSVT
A 26	GGGQIVGGVYLLPRR	A 104	EDGVNYATGNLPGCS	A 222	LHAPTSGSGKSTKVP	A 264	SILGIGTVLDOAET
A 27	GQIVGGVYLLPRRG	A 105	DGVNYATGNLPGCSF	A 223	LHAPTSGSGKSTKVP	A 265	SILGIGTVLDOAETA
A 28	QIVGGVYLLPRRGP	A 106	GVNYATGNLPGCSFS	A 224	HAPTSGSGKSTKVPAA	A 266	LIGIGTVLDOAETAG
A 29	QIVGGVYLLPRRGPR	A 107	VNYATGNLPGCSFSI	A 225	APTSGSGKSTKVPAA	A 267	GIGTVLDOAETAGVR
A 30	IVGGVYLLPRRGPRP	A 108	NYATGNLPGCSFSIF	A 226	PTSGSGKSTKVPAA	A 268	IGTVLDOAETAGVRL
A 31	VGGVYLLPRRGPRLG	A 109	YATGNLPGCSFSIFL	A 227	TSGSGKSTKVPAA	A 269	GTVLDOAETAGVRLT
A 32	GGVYLLPRRGPRLV	A 110	ATGNLPGCSFSIFLL	A 228	SGSGKSTKVPAA	A 270	VLDOAETAGVRLTV
A 33	GVYLLPRRGPRLVGR	A 111	TGNLPGCSFSIFLLA	A 229	SGKSTKVPAA	A 271	VLDOAETAGVRLTVL
A 34	VYLLPRRGPRLVGRV	A 112	GNLPGCSFSIFLLAL	A 230	GKSTKVPAA	A 272	LDQAETAGVRLTVLA
A 35	YLLPRRGPRLVGRAT	A 113	NLPGCSFSIFLLALL	A 231	KSTKVPAA	A 273	QAETAGVRLTVLAT
A 36	LLPRRGPRLVGRATR	A 114	LPGCSFSIFLLALLS	A 232	STKVPAA	A 274	QAETAGVRLTVLAT
A 37	LPRRGPRLVGRATRK	A 115	PGCSFSIFLLALLSC	A 233	TKVPAA	A 275	TAETAGVRLTVLATAT
A 38	PRRGPRLVGRATRKTR	A 116	QLINTNGSWHINRTA	A 234	KVPAA	A 276	ETAGVRLTVLATATP
A 39	RRGPRLVGRATRKTS	A 117	QLINTNGSWHINRTA	A 235	VPA	A 277	TAGVRLTVLATATPP
A 40	RGPRLVGRATRKTS	A 118	LINTNGSWHINRTAL	A 236	PA	A 278	AGVRLTVLATATPPG
A 41	GPRLVGRATRKTSER	A 119	INTNGSWHINRTALN	A 237	AA	A 279	GVRLTVLATATPPGS
A 42	RLGPRLVGRATRKTS	A 120	NTNGSWHINRTALNC	A 238	YA	A 280	VRLTVLATATPPGSV
A 43	RLGPRLVGRATRKTS	A 121	TNGSWHINRTALNCN	A 239	AA	B 1	SGMFDSSVLCCEYDA
A 44	LGPRLVGRATRKTS	A 122	NGSWHINRTALNCND	A 240	AA	B 2	GMFDSSVLCCEYDAG
A 45	GVPRLVGRATRKTS	A 123	GSWHINRTALNCNDS	A 241	AG	B 3	MFDSVLCCEYDAGC
A 46	VPRLVGRATRKTS	A 124	SWHINRTALNCNDL	A 242	Q	B 4	FDSSVLCCEYDAGCA
A 47	RATRKTSERSQPRGR	A 125	QLVNTNGSWHINRT	A 243	G	B 5	DSSVLCCEYDAGCAW
A 48	ATRKTSERSQPRGRR	A 126	QLVNTNGSWHINRTA	A 244	Y	B 6	SSVLCCEYDAGCAWY
A 49	TRKTSERSQPRGRRO	A 127	LVNTNGSWHINRTAL	A 245	K	B 7	SVLCCEYDAGCAWYE
A 50	RKTSERSQPRGRROP	A 128	VNTNGSWHINRTALN	A 246	V	B 8	VLCEYDAGCAWYEL
A 51	KTSERSQPRGRROP	A 129	VDYPYRLWHYPCTVNF	A 247	L	B 9	LCCEYDAGCAWYELT
A 52	TSERSQPRGRROP	A 130	DYPYRLWHYPCTVNF	A 248	V	B 10	CEYDAGCAWYELTP
A 53	SERSQPRGRROP	A 131	YPYRLWHYPCTVNF	A 249	Y	B 11	ECYDAGCAWYELTPA
A 54	DPRRSRNLGKVIDT	A 132	PYRLWHYPCTVNF	A 250	L	B 12	CYDAGCAWYELTPAE
A 55	PRRRSRNLGKVIDTL	A 133	YRLWHYPCTVNF	A 251	H	B 13	YDAGCAWYELTPAET
A 56	RRSRNLGKVIDTLT	A 134	RLWHYPCTVNF	A 252	A	B 14	DAGCAWYELTPAETT
A 57	RRSRNLGKVIDTLTC	A 135	LWHYPCTVNF	A 253	P	B 15	AGCAWYELTPAETT
A 58	RSRNLGKVIDTLTCG	A 136	WHYPCTVNF	A 254	T	B 16	GCAWYELTPAETT
A 59	SRNLGKVIDTLTCGFA	A 137	HYPCVNF	A 255	G	B 17	CAWYELTPAETT
A 60	RNLGKVIDTLTCGFA	A 138	YPCVNF	A 256	S	B 18	AWYELTPAETT
A 61	NLGKVIDTLTCGFA	A 139	PCTVNF	A 257	G	B 19	WYELTPAETT
A 62	LGKVIDTLTCGFA	A 140	CTVNF	A 258	K	B 20	YELTPAETT
A 63	GKVIDTLTCGFA	A 141	TVNF	A 259	S	B 21	ELTPAETT
A 64	KVIDTLTCGFA	A 142	VNF	A 260	T	B 22	LP
A 65	VIDTLTCGFA	A 143	NF	A 261	K	B 23	LP
A 66	OTLTCGFA	A 144	TF	A 262	V	B 24	LP
A 67	DTLTCGFA	A 145	TF	A 263	V	B 25	LP
A 68	TLTCGFA	A 146	IF	A 264	V	B 26	LP
A 69	LTTCGFA	A 147	DY	A 265	V	B 27	LP
A 70	TCGFA	A 148	Y	A 266	V	B 28	LP
A 71	CGFA	A 149	P	A 267	V	B 29	LP
A 72	GFA	A 150	Y	A 268	V	B 30	LP
A 73	FADLMGYIPV	A 151	RL	A 269	V	B 31	LP
A 74	ADLMGYIPV	A 152	L	A 270	V	B 32	LP
A 75	DLMGYIPV	A 153	W	A 271	V	B 33	LP
A 76	DPHRSRNVGKVIDT	A 154	H	A 272	V	B 34	LP
A 77	PHRSRNVGKVIDTL	A 155	Y	A 273	V	B 35	LP
A 78	HRSRNVGKVIDTLT	A 156	P	A 274	V	B 36	LP

Peptide ID	
B 41	CECYDAGAAWYELTP
B 42	ECYDAGAAWYELTPA
B 43	CYDAGAAWYELTPAE
B 44	YDAGAAWYELTPAET
B 45	DAGAAWYELTPAETT
B 46	AGAAWYELTPAETTV
B 47	GAAWYELTPAETTVR
B 48	AAWYELTPAETTVRL
B 49	DAGAAWYELTPAETS
B 50	AGAAWYELTPAETSV
B 51	GAAWYELTPAETSVR
B 52	AAWYELTPAETSVRL
B 53	FWAKHMMWNFISGIQY
B 54	WAKHMMWNFISGIQYL
B 55	AKHMMWNFISGIQYLA
B 56	KHMMWNFISGIQYLAG
B 57	HMMWNFISGIQYLAGL
B 58	MWNFISGIQYLAGLS
B 59	WNFISGIQYLAGLST
B 60	NFISGIQYLAGLSTL
B 61	FISGIQYLAGLSTLP
B 62	ISGIQYLAGLSTLPG
B 63	SGIQYLAGLSTLPGN
B 64	GIQYLAGLSTLPGNP
B 65	QYLAGLSTLPGNP
B 66	QYLAGLSTLPGNPAI
B 67	YLAGLSTLPGNPAIA
B 68	LAGLSTLPGNPAIAS
B 69	AGLSTLPGNPAIASL
B 70	GLSTLPGNPAIASLM
B 71	LSTLPGNPAIASLMA
B 72	STLPGNPAIASLMAF
B 73	QYLAGLSTLPGNPAV
B 74	YLAGLSTLPGNPAVA
B 75	LAGLSTLPGNPAVAS
B 76	AGLSTLPGNPAVASM
B 77	GLSTLPGNPAVASMM
B 78	LSTLPGNPAVASMMA
B 79	STLPGNPAVASMMAF
B 80	GAAGVSGIGKVLVD
B 81	AAVGSIGLKVLDI
B 82	AVGSIGLKVLDI
B 83	VGSIGLKVLDILA
B 84	GSIGLKVLDILAG
B 85	SIGLKVLDILAGY
B 86	GLKVLDILAGYG
B 87	GLKVLDILAGYGA
B 88	LGKVLDILAGYGAG
B 89	GKVLDILAGYGAGV
B 90	KVLDILAGYGAGVA
B 91	VLDILAGYGAGVAG
B 92	LDILAGYGAGVAGA
B 93	VDILAGYGAGVAGAL
B 94	DILAGYGAGVAGALV
B 95	LAGYGAGVAGALVA
B 96	AGYGAGVAGALVAF
B 97	GYGAGVAGALVAFK
B 98	YGAGVAGALVAFKI
B 99	GAGVAGALVAFKIM
B 100	GAGVAGALVAFKIMS
B 101	AGVAGALVAFKIMSG
B 102	GVAGALVAFKIMSGE
B 103	YGAGVAGALVAFKIM
B 104	YAGVAGALVAFKIMV
B 105	GAGVAGALVAFKIMS
B 106	AGVAGALVAFKIMSG
B 107	GVAGALVAFKIMSGE
B 108	GKVLVDILAGYGAGI
B 109	KVLVDILAGYGAGIS
B 110	VLVDILAGYGAGISG
B 111	LDILAGYGAGISGA
B 112	VDILAGYGAGISGAL
B 113	DILAGYGAGISGALV
B 114	LAGYGAGISGALVAF
B 115	AGYGAGISGALVAFK
B 116	YGAGISGALVAFKI
B 117	GYGAGISGALVAFKI
B 118	YAGISGALVAFKIM
B 119	GAGISGALVAFKIMS

Peptide ID	
B 120	AGISGALVAFKIMSG
B 121	GISGALVAFKIMSGE
B 122	VNLLPAILSPGALVV
B 123	NLLPAILSPGALVVG
B 124	LLPAILSPGALVVG
C 1	LPAILSPGALVVG
C 2	PAILSPGALVVG
C 3	AILSPGALVVG
C 4	ILSPGALVVG
C 5	LSPGALVVG
C 6	SPGALVVG
C 7	PGALVVG
C 8	GALVVG
C 9	ALVVG
C 10	LVVG
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C 12	VG
C 13	G
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C 71	R
C 72	L
C 73	V
C 74	V

Peptide ID	
C 75	FPDLGVRVCEKMALY
C 76	PDGVRVCEKMALYD
C 77	DLGVRVCEKMALYDV
C 78	GKGAARLIVYDPL
C 79	GGKGAARLIVYDPLG
C 80	GKGAARLIVYDPLGV
C 81	KGAARLIVYDPLGVR
C 82	KAARLIVYDPLGVRV
C 83	AARLIVYDPLGVRVC
C 84	ARLIVYDPLGVRVCE
C 85	RLIVYDPLGVRVCEK
C 86	LIVYDPLGVRVCEKM
C 87	IVYDPLGVRVCEKMA
C 88	VYDPLGVRVCEKMAL
C 89	YDPLGVRVCEKMALY
C 90	AQPGYPWPLYGNEGL
C 91	GQPGYPWPLYGNEGL
C 92	AFCSAMVYDGLCGSV
C 93	AFCALYVYDGLCGSV
C 94	ETVQDCNCSYPGHV
C 95	EFVQDCNCSYPGHV
C 96	GVLAGLAYSMVGNW
C 97	GVLAGLAYSMVGNW
C 98	QDRPYCWHYAPRPGC
C 99	QDRPYCWHYAPRPGC
C 100	TCPTDCFRKHEPATY
C 101	YTKGSGPWLTTPRCL
C 102	LNAACNWTGRGERCDL
C 103	LNAACNWTGRGERCDL
C 104	IAQAEAALENLVN
C 105	IAQAEAALEKLVN
C 106	TRVPYFVRAQGLIRA
C 107	TRVPYFVRAHGLIRA
C 108	HAGLRDLAVAVEPV
C 109	AAGLRDLAVAVEPIV
C 110	ITWGADTAACGDIL
C 111	ITWGAETAACGDIL
C 112	GQGWRLAFITAYSQ
C 113	TAYSQQTGRLGCI
C 114	TAYSQQTGRLGCI
C 115	GCIITSLTGRDKNQV
C 116	GCIIVSMTGRDKTQV
C 117	VNGVCWTVYHAGGSK
C 118	KGPITQMYTNVDQDL
C 119	KGPITQMYSSAEQDL
C 120	GDSRGSLSPRPVSY
C 121	GDSRGALLSPRPVSY
C 122	SYLKGSSGGPLCP
C 123	SYLKGSSGGPVLCP
C 124	GHAVGIFRAAVCTRG
C 125	GVDPNIRTGVRTIT
C 126	VPHNPINEVALSNTG
C 127	TGEIPFYGKAIPV
C 128	TGEIPFYGKAIPLEV
C 129	PTSGDVVATDAML
C 130	QTVDFSLDPTTET
C 131	TLHGPTPLLYRLGAV
C 132	VQNEVTLTHPTIKY
C 133	LYREFDEMEECASHL
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C 140	VAAEEYEVTRHGD
C 141	FFTEVDGVRHRYAP
C 142	FFTELDGVRHRYAP
C 143	FFTWVDGVIHRYAP
C 144	FFTWLDGVIHRYAP
C 145	YLVGSQPCPEPDV
C 146	YLVGSQPCPEPDV
C 147	LPWARPDPNPLLE
C 148	ASLRQKVTDFRLQV
C 149	ASLRQKVTDFRLQV
C 150	HIRSVWKDLEDDET
C 151	DTTIMAKNEVFCVQ
C 152	VMGSSYGFQYSPGQR
C 153	DCTMLVCGDDLWVIC

Peptide ID	
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C 155	DPTMLVCGDDLWVIC
C 156	DPTMLVCGDDLWVIC
C 157	LWARMILMTHFFSIL
C 158	LWVRMMLMTHFFSIL
C 159	DLQNIERLHGLSAF
C 160	DLQNIERLHGLSAF
C 161	AVRTKLKLTPIPAAS
C 162	AVRTKLKLTPIPAAS
C 163	SGGDYHSLSRARPR
C 164	SGGDYHSLSRARPR
C 165	WGENETDVLNNTNR
C 166	GENETDVLNNTNR
C 167	WFGCTWMNSTGFTKT
C 168	FCTWMNSTGFTKT
C 169	GCTWMNSTGFTKT
C 170	GLPVSARRGREILLG
C 171	LPVSARRGREILLGP
C 172	PVSARRGREILLGPA
C 173	VSARRGREILLGPA
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Peptide ID (pep)	
1506	MSTNPKPQRKTKRNTNRRPQDKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRGRQPIPK
1526	VNLLPAILSPGALVGVVCAAILRRHVGPGEQAVQWMNRLIAFASRGNHVSPHYV
1545	IKGGRHLIFCHSKKKCELA
1546	TVPQDAVSRSQRRGRGTGRGR
1547	YLVAYQATVCARAQAPPSWD
1551	HLHAPTSGSKSTKVPAAQGYKVLNPSVAATLGFAY
1552	YLHAPTSGSKSTKVPVAAQGYKVLNPSVAATLGFAY
1553	GAAVGSIGLGKVLVDILAGYGAGVAGALVAFKIMSGE
1554	GAAVGSIGLGKVLVDILAGYGAGVAGALVAFKIMSGE
1555	GAAVGSIGLGKVLVDILAGYGAGISGALVAFKIMSGE
1556	FTEAMTRYSAAPPDPP
1557	SSMPLEGEPPDPL
1558	CGYRRCRASGLTTS
1559	PVNSWLGNHMYAPT
1560	PVNSWLGNHMYAPT
1561	SGMFDSSVLCCEYDAGCAWYELTPAETTVRLRAY
1562	SGMFDSSVLCCEYDAGCAWYELTPAETSVRLRAY
1563	SGMFDSSVLCCEYDAGCAWYELTPAETTVRLRAY
1564	SGMFDSSVLCCEYDAGCAWYELTPAETSVRLRAY
1565	FWAKHMWNFISGIQYLAGLSTLPGNPAIASLMAF
1577	GEVQVSTATQSFLAT
1578	GEVQVSTVTQSFLGT
1579	FTDNSSPPAVPQTFQV
1580	FSDNSTPPAVPQTYQV
1581	NAVAYYRGLDVSPIPT
1587	VNLLPGILSPGALVGVVCAAILRRHVGPGEQAVQWMNRLIAFASRGNHVAPHYV
1588	ITILGIGTVLDQAETAGARLVLATATPPGSVT
1589	TSILGIGTVLDQAETAGARLVLATATPPGSVT
1590	TSILGIGTVLDQAETAGVRLTVLATATPPGSVT
1591	ITILGIGTVLDQAETAGVRLTVLATATPPGSVT
1592	FWAKHMWNFISGIQYLAGLSTLPGNPAVASMMMAF
1603	VFTGLTHIDAHFLSQTKQ
1604	VCCSMSYTWGALITPC
1605	VCCSMSYTWGALITPC
1606	VLTSMLTDPSHITAETA
1607	VLTSMLTDPSHITAETA
1613	ASSSASQLSAPSLRATCTT
1614	LTPPHSARSKFGYGAKEVR
1615	LTPPHSARSKFGYGAKEVR
1616	LTPPHSARSKYGYGAKEVR
1617	LTPPHSARSKYGYGAKEVR
1618	PMGFSDYTRCFDSTVTE
1619	PMGFAYDTRCFDSTVTE
1620	TGDFDSVIDCNVAVTQ
1621	TGDFDSVIDCNVAVTQ
1622	NTPGLPVCQDHLFEWE
1623	YLVAYQATVCARAQAPPSWD
1624	LEDRLRSQSLPLHSTTEW
1625	LEDRLRSQSLPLHSTTEW
1626	ASSSASQLSAPSLKATCTT
1627	PEYDLELITSCSSNVSA
1628	VCGPVYCTPSPVVGTDDR
1629	GWAGWLLSPRGRPSWGP
1630	LLFLLADARVCACLWM
1631	SGHRMAWDMMNWSPT
1632	TGHRMAWDMMNWSPT
1641	ITYSTYKFLADGGCSGGAYDIIICDECHS
1647	ARALAHGVRVLEDGVNYATGNLPGCSFIFLLALLSC
1648	DPRRRSRNLGKVIDLTCGFADLMGYPLVGAPLGG
1649	DPRRRSRNLGKVIDLTCGFADLMGYPLVGAPLGG
1650	VDYPYRLWHYPCTVNTIFKIRMYVGGVEHRL
1651	VDYPYRLWHYPCTVNTIFKIRMYVGGVEHRL
1652	XGGRKPARLIVFDLGVVCEKMALYDV
1653	XGGRKPARLIVFDLGVVCEKMALYDV
1654	IQLVNTNGSWHINRTALNCNDSL
1655	IQLVNTNGSWHINRTALNCNDSL
1656	LPALSTGLIHLQNVQVQYLYG

For epitope capture, each peptide pool was incubated with soluble recombinant HLA-class II molecules and specific binding was assessed by an SDS-stability assay. The results using the HLA molecules DRB1*0401, DRB1*0404 and DRB1*0101 are shown in Fig. 2 and 3 respectively: 28 peptide pools were found which bind to DRB1*0401 molecules: no. 1, 2, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20 from "row" pools and no. 23, 25, 26, 27, 29, 30, 31, 34, 36, 38, 39 and 40 from "column" pools (Fig. 2). 35 peptide pools out of 40 tested were positive in binding to DRB1*0404 molecules (Fig. 3), while all peptide pools showed binding activity to DRB1*0101 molecules. By finding the intersections of reactive pools in the array, potential individual binders were determined and re-checked for binding affinity individually.

All individually confirmed peptides are summarized in Table 2. Binding to DRB1*0401 is shown in Fig. 4: 54 individual peptides were identified as ligands of this HLA-type. Often several overlapping 15mers in a row bound to HLA allowing identification of their core binding regions. Peptide differing only by one or two amino acids representing variants (see Table 1) usually bound both to soluble HLA class II molecules. Such "duplicates" were considered to represent the same epitope. Thus, 31 ligands capable to bind to DRB1*0401 were identified, including 11 previously known class II epitopes. From the latter, however, only two (A202-A206 and B60-B68) had been known to be restricted to DR4 (see Table 2). 20 ligands are candidates for novel epitopes. For DRB1*0404, 64 binders designated as 28 potential epitopes were determined, 4 of them belong to already known epitopes (Fig. 5, Table 2). For DRB1*0101, 83 peptides representing 44 potential epitopes were identified (Fig. 6, Table 2). Of those, 7 had been described previously but with different HLA restriction.

All individually confirmed peptides binding to at least one of the 3 above mentioned HLA types were also tested for affinity to DRB1*0701 molecules in a peptide-competition assay (Fig. 7, Table 2). Here, 50 ligands were identified. Of those, 7 correspond to already known class II epitopes, but only one was described as DRB1*0701epitope (A202-A206).

Table 2. HCV derived peptides binding to soluble HLA class II molecules. About 400 15- to 23-mer peptides derived from conserved regions of HCV were analyzed by the Epitope Capture Method using pools of up to 20 peptides arrayed in matrix format (see Fig. 1) and four different HLA class II molecules. Specific binding was confirmed for individual peptides.

ID	Peptide sequence	Binding to DRB1				Known / new potential epitope, HLA coverage
		*0101	*0401	*0404	*0701	
A120	NTNGSWHINRTALNC	*			nb	new DRB1*0101, *0401, *0404, *0701: 45-55%
A122	NGSWHINRTALNCND	*			*	
A124	SWHINRTALNCNDSL	*			*	
B25	DAGCAWYELTPAETS	***	***	***	nb	new DRB1*0101, *0401, *0404, *0701: 45-55%
B26	AGCAWYELTPAETSV	***	***	***	*	
B28	CAWYELTPAETSVRL	***	**	***	*	
B30	WYELTPAETSVRLRA	**	*	***	nb	
B46	AGAAWYELTPAETTV	***		***	nb	new DRB1*0101, *0401, *0404, *0701: 45-55%
B48	AAWYELTPAETTVRL	***		***	**	
B84	GSIGLGKVLVDILAG	*	*	*	*	new DRB1*0101, *0401, *0404, *0701: 45-55%
B86	IGLGKVLVDILAGYG	*	**	*	**	
B88	LKGVLVDILAGYGAG	*	**	*	*	
B92	LYDILAGYGAGVAGA	*	nb	*	*	
C106	TRVPYFVRAQGLIRA	*	*	*	*	new DRB1*0101, *0401, *0404, *0701: 45-55%
C113	TAYSQQTRGLLGCI	***	*	*	*	
C114	TAYSQQTRGLLGCIIV	***	*	*	*	
1627	PEYDLELITSCSSNVSA	***		***	**	new DRB1*0101, *0401, *0404, *0701: 45-55%
1628	VCGPVYCFTPSPVVGTTDR	***	**	*	*	
1629	GWAGWLLSPRGRPSWGP	*	*	**	*	
1604	VVCCSMSYWTGALITPC	*		**	***	new DRB1*0101, *0401, *0404, *0701: 45-55%
1630	LLFLLADARVCACLWM	*		nb	***	new DRB1*0101, *0401, *0701: 40-50%
C97	GVLFGLAYFSMVGNW	**	**	nb	**	
1547	YLVAYQATVCARACAPPSWD	***		nb	**	
B94	DILAGYGAGVAGALV	*	nb	nb	nb	new DRB1*0101, *0401, *0701: 40-50%
B95	LAGYGAGVAGALVA	*	nb	nb	*	
B96	LAGYGAGVAGALVAF	**	**	nb	*	
B97	AGYGAGVAGALVAFK	*	nb	nb	*	
B98	GYGAGVAGALVAFKI	*	nb	nb	nb	
A272	ETAGVRLTVLATATP	*	*	nb	*	new DRB1*0101, *0401, *0701: 40-50%
A274	AGVRLTVLATATPPG	*	nb	*	nb	
A276	VRLTVLATATPPGSV	*	nb	*	*	
B120	AGISGALVAFKIMSG	*	nb	*	***	new DRB1*0101, *0404, *0701: ~45%
B122	VNLLPAILSPGALVV	*	nb	*	*	
C108	HAGLRDLAVAVEPVV	*	nb	*	*	
C134	ITLLFNILGGWVAAQ	**	nb	*	**	new DRB1*0101, *0404, *0701: ~45%
C152	VMGSSYGFQYSPGQR	*	nb	*	*	
1606	VLTSMLTDPSSHITAETA	nb	***	**	**	new DRB1*0401, *0404, *0701: ~45%
1607	VLTSMLTDPSSHITAEAA	nb	***	**	*	
1577	GEVQVVSTATQSFLAT	nb	*	***	***	new DRB1*0401, *0404, *0701: ~45%
1578	GEVQVLSTVTQSFLGT	nb	*	***	***	
B50	AGAAWYELTPAETSV	***	***	***	nb	new DRB1*0101, *0401, *0404: ~40%
B52	AAWYELTPAETSVRL	***	***	***	nb	
1623	YLVAYQATVCARACAPPSWD	*		**	*	new DRB1*0101, *0401, *0404: ~40%
C130	QTVDFSLDPTFTIET	**	***	**	nb	
1603	VFTGLTHIDAHFLSQTKQ	***	nb	nb	*	new DRB1*0101, *0701: ~40%
C96	GVLAGLAYYSMVGNW	**	nb	nb	*	

C191	YYLTRDPTIPLARAA	nb	***	nb	*	new DRB1*0401, *0701:	~40%
A216	SGKSTKVPVAYAAQG	nb	*	nb	nb		
A218	KSTKVPVAYAAQGYK	nb	*	nb	nb		
A220	TKVPVAYAAQGYKVL	*	**	nb	nb	new DRB1*0101, *0401:	~35%
A222	VPVAYAAQGYKVLVL	*	nb	nb	nb		
A224	VAYAAQGYKVLVLNP	*	nb	nb			
A242	TILGIGTVLDQETA	nb	nb	*			
A244	LGIGTVLDQETAGA	*	*	nb	nb	new DRB1*0101, *0401:	~35%
C92	AFCSAMYVGDLCGSV	*	**	nb	nb	new DRB1*0101, *0401:	~35%
C93	AFCSALYVGDLCGSV	*	*	nb			
A174	PALSTGLLHLHQNIV		nb	*	*	new DRB1*0404, *0701:	25-30%
B32	SGMFDVVLCCECYDA	*	nb	***			
B34	MFDSVVLCCECYDAGA	*	nb	***	nb	new DRB1*0101, *0404:	20-25%
B36	DSVVLCCECYDAGAAW	*	nb	***			
B38	VVLCCECYDAGAAWYE	nb	nb	*	nb		
B100	GAGVAGALVAFKIMS	**	nb	**		new DRB1*0101, *0404:	20-25%
B102	GVAGALVAFKIMSGE	***	nb	***			
C135	ITLLNLGGWLAQAQ	**	nb	*		new DRB1*0101, *0404:	20-25%
C162	AVRTKLKLTPLAAS	nb	*	*	nb	new DRB1*0401, *0404:	20-25%
1618	PMGFSYDTRCFDSTVTE	nb	nb	**		new DRB1*0701:	~25%
1622	NTPGLPVCQDHLEFWE	nb	nb	***		new DRB1*0701:	~25%
1624	LEDRDRSELSPILLSTTEW	nb	nb	*		new DRB1*0701:	~25%
1546	IVPQDAVSRQRGRGTGRGR	nb	nb	nb	*	new DRB1*0701:	~25%
1556	FTEAMTRYSPPGDPP	nb	nb	nb	*	new DRB1*0701:	~25%
A114	LPGCSFSIFLLALLS	**	nb	nb	nb	new DRB1*0101:	~20%
B58	MWNFISGIQYLAGLS	*	nb	nb		new DRB1*0101:	~20%
B112	VDILAGYGAGISGAL	*	nb				
B114	ILAGYGAGISGALVA	***	nb			new DRB1*0101:	~20%
B116	AGYGAGISGALVAFK	***	nb	nb	nb		
B118	YGAGISGALVAFKIM	***	nb	nb			
B18	DAGCAWYELTPAETT	***	nb	nb	nb		
B20	GCAWYELTPAETTVR	***	nb			new DRB1*0101:	~20%
B22	AWYELTPAETTVRLR	**	nb	nb	nb		
C112	GQGWRLAPITAYSQ	**	nb			new DRB1*0101:	~20%
C116	GCIVVSMTRDKTQV	*	nb	nb		new DRB1*0101:	~20%
C122	SYLKGSSEGGPLLCP	*	nb	nb		new DRB1*0101:	~20%
C127	TGEIPFYGKAIEV	*	nb			new DRB1*0101:	~20%
C144	FFTWLDGVQIHRYP	**	nb	nb		new DRB1*0101:	~20%
C159	DLPQIIRLHGLSAF	*	nb	nb	nb	new DRB1*0101:	~20%
C160	DLPQIIRLHGLSAF	*	nb				
C174	GLPVSALRGREILLG	*	nb	nb		new DRB1*0101:	~20%
1558	CGYRRCRASGVLTT	***	nb	nb	nb	new DRB1*0101:	~20%
1581	NAVAYYRGLDVSVIPT	**	nb	nb	nb	new DRB1*0101:	~20%
C95	EFVQDCNCSIYPGHV	nb	**	nb	nb	new DRB1*0401:	~20%
C129	PTSGDVVVVATDALM	nb	nb	**		new DRB1*0404:	~5%
C157	LWARMILMTHFFSIL	nb	nb	*	nb	new DRB1*0404:	~5%
C158	LWVRMVLMTTHFFSIL		nb	*			
A254	ETAGARLVVLTATP	nb	nb	*			
A256	AGARLVVLTATPPG	nb	nb	*		new DRB1*0404:	~5%
A258	ARLVVLTATPPGSV	nb	nb	**			
1605	VVCCSMSYSWTGALITPC	nb	nb	*	nb	new DRB1*0404:	~5%
C109	AAGLRDLAVAVEPIV		nb	*		new DRB1*0404:	~5%
C161	AVRTKLKLTPIPAAS		nb	*		new DRB1*0404:	~5%
A60	LQKWDITLCGFA	**	nb	nb	**	known DR4, DR8, DR15	

A61	GKVIDTLTCGFAD	**				new DR*0101, 0701
A70	TCGFADLMGYIPLVG	*	nb	nb		
A72	GFADLMGYIPLVGAP	***	*	**		known class II
A74	ADLMGYIPLVGAPLG	***		***	**	DR*0101, 0404, 0701
A88	CGFADLMGYIPVVGGA	**		**	*	
A90	FADLMGYIPVVGAPL	***		***		known class II
A92	DLMGYIPVVGAPLGG	***		***	***	DR*0101, 0404, 0701
A96	LAHGVRVLEDGVNYA	nb		nb		
A98	HGVRVLEDGVNYATG	nb	***		**	known DR11
A100	VRVLEDGVNYATGNL	nb	***	***	*	new DR*0401, 0404, 0701
A102	VLEDGVNYATGNLPG	nb		*		
A104	EDGVNYATGNLPGCS	nb	*	nb	nb	
A200	AAQGYKVLVNLNPSVA		nb	***	**	
A202	QGYKVLVNLNPSVAAT	***	***	***	***	known DRB1*0401, 0701, DR11, DR15
A204	YKVLVNLNPSVAATLG	***	***	***	***	new DR*0101
A206	VLVNLNPSVAATLGFG	***	***	***	***	
C30	AVQWMNRLIAFASRG	*	nb	nb		known DR11, DQ5, also DR*0101
B60	NFISGIQYLAGLSTL	**		nb	*	
B62	ISGIQYLAGLSTLPG	***		***	**	
B64	GIQYLAGLSTLPGNP	***		***	**	known DR*0401, 1101
B66	QYLAGLSTLPGNPPI	*		***	**	new 0101, 0404, 0701
B68	LAGLSTLPGNPPIAS	*		***	**	
C124	GHAVGIFRAAVCTRG	**	*	nb	**	known DR*0101, 0401, 0701
I620	TGDFDSVIDCNTCVTQ	nb	nb	*		new DR*0404
I621	TGDFDSVIDCNVAVTQ	*		nb	nb	known DR13, also DR*0101, 0401
I631	SGHRMAWDMMMNWSPT	nb		nb		known class II, also DR*0401
I632	TGHRMAWDMMMNWSPT	nb	*			known class II, also DR*0401

*** strong binding
 ** intermediate binding
 * weak binding
 nb no binding

Boldface peptide IDs indicate HLA-ligands with confirmed immunogenicity in HLA-transgenic mice

Boldface peptide sequences indicate putative core binding regions based on prediction algorithms as described in the text.

¹⁾ immunogenic in DRB1*0401 transgenic mice

Some of the highly promiscuous peptides and/or with computer algorithm (SYFPEITHI, TEPITOPE)-predicted affinities were checked for binding to soluble HLA-DRB1*1101 molecules in a peptide-competition assay as it is described for HLA-DRB1*0701. Several known DR11 epitopes were used as controls and were confirmed to bind HLA-DRB1*1101 molecules in vitro. Among newly identified HLA-DRB1*1101 binders, there are peptides with IDs A120, A122, A141, C114, C134, 1426, 1628, 1629 of high affinity, 5 peptides with IDs C106, C135, 1578, 1547, 1604 of moderate affinity and 4 peptides with IDs B46, B48, B86, B96 of weak affinity ligands.

In summary eight novel ligands binding at least to HLA-DRB1*0101, *0401, *0404, *0701 and *1101 (Tab. 2: peptide IDs A120, A122, A141, 1604, 1547, 1628, 1629, and Tab. 6: peptide ID 1426); novel 10 ligands binding at least to HLA-DRB1*0101, *0401, *0404 and *0701 (Tab. 2: peptide IDs A120-A124, B25-B30, B46-B48, B84-B92, C106, C113-C114, 1627, 1628, 1629, 1604); 5 novel ligands binding at least to HLA-DRB1*0101, *0401 and *0701, 5 novel ligands binding at least to HLA-DRB1*0101, *0404 and *0701, 4 novel ligands binding at least to HLA-DRB1*0401, *0404 and *0701, 3 novel ligands binding at least to HLA-DRB1*0101, *0401 and *0404, 2 novel ligands binding at least to HLA-DRB1*0101 and *0701, 1 novel ligand binding at least to HLA-DRB1*0401 and *0701, 3 novel ligands binding at least to HLA-DRB1*0101, *0401, 1 novel ligand binding at least to HLA-DRB1*0404 and *0701, 4 novel ligand binding at least to HLA-DRB1*0101 and *0404, 5 novel ligands binding at least to HLA-DRB1*0701, 13 novel ligands binding at least to HLA-DRB1*0101, 1 novel ligand binding at least to HLA-DRB1*0401, and 6 novel ligands binding at least to HLA-DRB1*0404.

Moreover, 12 known HLA class II epitopes were confirmed, in several cases binding to alleles not reported yet was demonstrated (Tab. 2, last group).

Having established physical binding too HLA class II it is straightforward to verify immunogenicity for a given ligand: for instance peptide IDs A120-A124, B46-B48, 1627, 1604, 1630, 1547, 1623, B112-118, 1558, all binding to one or more HLA class II alleles were also shown to be immunogenic in HLA-DRB1*0401

transgenic mice (see Example II).

To determine the optimal epitope within a longer polypeptide, mice can be vaccinated with a longer polypeptide incorporating the candidate epitope sequences. Generation of specific CD4+ T cell responses against naturally processed and presented epitopes can then be assayed by re-stimulation of murine splenocytes or lymph node cells with overlapping 15-mers and IFN-gamma ELISpot. Final confirmation/validation of the newly identified HLA-ligands can be achieved by testing these peptides with T-cells from humans. Ideally, these comprise therapy responders or subjects spontaneously recovered from infection.

Example II. Immunogenicity of HCV-derived peptides in HLA-transgenic mice

Synthetic HCV-derived peptides (from conserved regions) were investigated for immunogenicity in HLA-transgenic mice: 36 of 68 peptides tested were found to induce peptide-specific IFN-gamma-producing cells in vaccination experiments. As summarized in Table 3, some peptides were either immunogenic (+, less than 100 peptide-specific cells among a million splenocytes) or even strongly immunogenic (+++, more than 100 peptide-specific cells among a million splenocytes) in DR4- and/or A*0201-transgenic mice.

Table 3:

Ipep DRB1*0401 A*0201 B*0702 Sequence				
1506			+	MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQ- PRGRRQPIPK
1526	+	+	+	VNLLPAILSPGALVVGVCAILRRHVGPGEQAVQWMNRLIAFASRGMHVSPTHYV
1545			+	IKGGRHLIFCHSKKCELA
1547	++		+++	YLVAYQATVCARAQAPPPSWD
1552		+	+	YLHPTGSGKSTKVPVAYAAQGYKVLVLPNSVAATLGFGAY
1553		+	+	GAAVGSIGLGKVLVDILAGYGAGVAGALVAFKIMSGE
1555	+	+	+	GAAVGSIGLGKVLVDILAGYGAGISGALVAFKIMSGE

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1558	++	+	++	CGYRRCRASGVLTS
1559	+	++		PVNSWLGNIIMYAPT
1560	+	++		PVNSWLGNIQYAPT
1562			+	SGMFDSSVLCCECYDAGCANYELTPAETSVRLRAY
1563	+		+	SGMFDSSVLCCECYDAGAAWYELTPAETTVRLRAY
1565	++	++	+	FWAKHMWNFISGIQYLAGLSTLPGNPAIASLMAF
1577		+	+	GEVQVVSTATQSFLAT
1578			+	GEVQVLSTVTQSFLGT
1580			+	FSDNSTPPAVPQTYQV
1587		+	+	VNLLPGILSPGALVVGVICAILRRHVGPGEQAVQWMNRLIAPASRGNEVAPTHYV
1592	++	++	+	FWAKHMWNFISGIQYLAGLSTLPGNPAVASMMAF
1604	++	++	++	VVCCSMSYTWGALITPC
1605	+	+	+	VVCCSMSYSWIGALITPC
1615		+		LTPPHSAKSKPGYGAKDVR
1616	+			LTPPHSAKSKYGYGAKEVR
1617		+		LTPPHSARSKYGYGAKEVR
1621	+	+	+	TGDFDSVIDCNVAVTQ
1623	+	+	+	YLWAYQATVCARAKAPPSWD
1624			+	LEDRDRSELSPLLSTTEW
1625	+			LEDRDRSQLSPLLSTTEW
1627	+	+	++	PETDLELITSCSSNVSA
1628			++	VCQFVYCFTSPVVGTTDR
1630	++	++		LLFLLADARVCACIWM
1631	+	+		SGHRMAWDMMNWSPT
1632		+		TGHRMAWDMMNWSPT
1641		+		ITYSTYCKFLADGGCSCGAYDIIICDECHS
1647		+	+	ARALAHGVRVLEDGVNYATGNLPGCSFSIFLLALLSC
1649	+			DPRHRSRNVGKVIDTLTCGFADLMGYIPVVGAPLGG
1650	++	++	+	VDYFYRLWHYPCTVNFTIFKIRMYVGGVEHRL
1651	++	++	+	VDYFYRLWHYPCTVNFTIFKIRMYVGGVEHRL
1652	++	+		KGGKPARLIVFPDLGVRVCEKMAIDV
1653		+		KGGKKAARLIVFPDLGVRVCEKMAIDV
1654	++	+		IQLINTNGSWHINRTALNCNDSL
1655	++	+		IQLVNTNGSWHINRTALNCNDSL
1656	+			LPALSTGLIHLHQNIVDYQYLYG

Peptide 1526, 1565, 1631, also shown to be immunogenic in HLA-DRB1*0401 transgenic mice contain known class II epitopes.

Peptide IDs 1526, 1553, 1565, 1587, 1623, 1630 also shown to be

immunogenic in HLA-A*0201 transgenic mice contain known A2 epitopes.

For further characterizing the novel epitopes provided herewith, one may define the exact HLA restriction of these epitopes and the minimal epitopes within the sequences recognized by T cells. Both can be done by a variety of well-established approaches known to the one skilled in the art (Current Protocols in Immunology, John Wiley & Sons, Inc.).

First, publicly available programs can be used to predict T cell epitopes on the basis of binding motifs. These include for instance: http://bimas.dcrt.nih.gov/molbio/hla_bind/ (Parker et al. 1994), <http://134.2.96.221/scripts/MHCServer.dll/home.htm> (Rammensee et al. 1999), <http://mypage.ihost.com/usinet.hamme76/> (Sturniolo et al. 1999). The latter prediction algorithm offers the possibility to identify promiscuous T helper-epitopes, i.e. peptides that bind to several HLA class II molecules. These predictions can be verified by testing of binding of the peptide to the respective HLA.

A way of quickly discerning whether the response towards a peptide is class I or class II restricted is to repeat the ELISpot assay with pure CD4+ or CD8+ T cell effector populations. This can for instance be achieved by isolation of the respective subset by means of magnetic cell sorting. Pure CD8+ T cells can also be tested in ELISpot assays together with artificial antigen-presenting-cells, expressing only one HLA molecule of interest. One example are HLA-A*0201 positive T2 cells (174CEM.T2, Nijman et al., 1993). Alternatively, one can use ELISpot assays with whole PBMCs in the presence of monoclonal antibodies specifically blocking either the CD4+ or CD8+ T cell sub-population. Exact HLA restriction can be determined in a similar way, using blocking monoclonal antibodies specific for a certain allele. For example the response against an HLA-A24 restricted epitope can be specifically blocked by addition of an HLA-A24 specific monoclonal antibody.

For definition of the minimal epitopes within the peptide sequences recognized by T cells, one can test overlapping and

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truncated peptides (e.g. 8-, 9-, 10-mers) with splenocytes from immunized transgenic mice or T-cells from humans recognizing the respective epitope.

Example III. HLA restriction of immunogenic HCV-derived peptides investigated in transgenic mice.

Groups of 5 mice (HLA-A*0201-, HLA-DRB1*0401- and HLA-B*0702 transgenic mice, male, 8-14 weeks of age) were injected subcutaneous into the hind footpads with 100µg of peptide + IC31 per mouse (50µg per footpad). (PCT/EP01/12041, WO 02/32451 A1 and PCT/EP01/06433, WO 01/93905 A1; IC31 is a combination of the immunizer disclosed in WO 01/93905 and WO 02/32451).

6 days after vaccination single cell suspension of pooled spleens were prepared and additionally pure fractions of CD8+ in the case of A2 and B7 tg mice (CD8+ fraction for B7 mice containing 97% of CD8 and 1.5% of CD4 cells and for A2 tg mice 83% of CD8 and 8% of CD4 cells) and CD4+ for DR4tg mice (CD4+ fraction for DR4tg mice containing 98% of CD4 cells and 0.2 % of CD8 cells) were separated from the spleen cell suspension using MACS separating kit (Miltenyi, Germany). All cells (not separated cells, positive and corresponding negative fractions) were restimulated ex vivo with relevant peptide (for instance Ipep1604) and irrelevant peptides as negative control (known HLA-DRB1*0401 CMV-derived epitope Ipep 1505, HLA-B*0702 HIV-derived epitope Ipep 1787, or HLA-A*0201 tyrosinase-derived epitope Ipep1124) to detect INF-γ-producing cells in ELISpot assay.

As an example shown in Fig. 8-10 the Ipep1604 (VVCCSMSYTWGALITPC, in combination with immunizer IC31) was able to induce high numbers of specific INF-γ-producing T cells in all three transgenic class I and II mouse strains. This was shown not only with whole spleen derived cells but also with enriched fractions of CD8+ cells correspondingly for A2 and B7 and CD4+ cells for DR4tg mice. Similar, albeit weaker responses were seen with Ipep1605 (VVCCSMSYSWTGALITPC), a sequence variant with a serine instead of a threonine.

Thus, Ipep1604 contains class I epitopes for HLA-A*0201 and HLA-B*0702 and a class II epitope for HLA-DRB1*0401 molecules.

As shown in Tables 2 and 6, Ipep 1604 binds to class II molecules in a promiscuous manner. Thus, it contains further epitopes, at least for HLA-DRB1*0101, DRB1*0404, DRB1*0701 and DRB1*1101.

Other peptides were analysed in a similar way:

Ipeps 1605, 1623, 1547, 1558, 1559, 1560, 1565, 1592, 1650, 1654 and 1655 were confirmed to contain human HLA-DRB1*0401 epitopes. Again, for most of these epitopes binding is not limited to HLA-DRB1*0401 as shown in Tables 2 and 6.

Ipeps 1565, 1605 and 1650 were confirmed to contain human HLA-A*0201 epitopes.

Ipeps 1506, 1587 were confirmed to contain human HLA-B*0702 epitopes.

Ipep 1843 with sequence LPRRGPRRL was shown to be the HLA-B*0702 minimal epitope contained in 1506:

Fig. 10 shows mouse IFN-gamma ELISPOT with splenocytes or separated CD8+ or CD4+ cells from HLA-A*0702 transgenic mice vaccinated with Ipep1506+IC31 or Ipep1835+IC31.

Fig. 10 A) and B) shows that after a single vaccination with either Ipep 1506+IC31 or Ipep1835+IC31, upon restimulation with overlapping 15mers, the 15mers A30 to A37 (see Tab.1) react. The common sequence of these 15mers is LPRRGPRRL (Ipep 1843, see Tab.4).

Fig. 10 C) confirms these findings: after a single vaccination with either Ipep1506+IC31 or Ipep1835+IC31, significant interferon-gamma induction against Ipep1843 can be detected. In both cases Ipep 1790 an HIV NEF-derived HLA-B*0702 epitope (sequence RPMTYKAAL was used as negative control for restimulation.

Ipep 1838 with sequence SPGALVVGVI (see Tab.4) was shown to be an HLA-B*0702 minimal epitope contained in 1587:

In the case of Ipep1587 a different approach was taken: the sequence of Ipep1587 was inspected for HLA-B*0702 binding motifs and a couple of short peptides were synthesized accordingly.

These were tested in a competition-type peptide binding assay using soluble HLA-B*0702 and the FITC-labelled reference peptide LPCVLWPFVL, which is a known HLA-B*0702 epitope derived from EBV (Stuber et al., 1995). Peptide Ipep1838 showed ~30% competition when used in 80-fold molar excess for 48h at 37°C. Thus it is likely to present the minimal HLA-B*0702 epitope contained in Ipep 1587.

Example IV: Identification and confirmation of novel HCV peptides reactive in IFN-gamma ELISpot with human PBMC from HCV therapy responders or patients with spontaneous recovery

40 peptide mixtures in matrix format (Fig. 1) containing synthetic peptides derived from conserved regions of HCV (Table 1) were screened in IFN-gamma ELISpot using PBMC from more than 50 individuals who were either responders to interferon/ribavirin standard therapy, or, who had spontaneously cleared HCV (i.e. all subjects were HCV antibody positive, but HCV-RNA negative). PBMC from such individuals are supposed to contain the relevant T-cell populations responsible for clearing HCV. Thus, peptides discovered or confirmed by using these PBMC are likely to represent the structural determinants of immune protection against/clearance of HCV. Based on the results from this primary matrix-screen, a number of peptides were chosen for individual re-testing in IFN-gamma ELISpot using PBMC from selected donors. In addition, several new peptides incorporating sequences from overlapping reactive peptides or avoiding critical residues like cysteine were synthesized. These are summarized in Table 4.

Table 4: additional peptides derived from conserved regions of HCV.

Peptide ID	Peptide sequence (1 amino acid code)
1006	MWNFISGIQYLAGLSTLPGN
1334	HMWNFISGI
1425	NFISGIQYLAGLSTLPGNPA
1426	HMWNFISGIQYLAGLSTLPGNPA
1798	IGLGKVLVDILAGYGAGVAGALVAFK
1799	AAWYELTPAETTVRLR
1800	DYPYRLWHYPCTVNYTIFKI
1836	DYPYRLWHYPCTVNFTIFKI

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1801	AYSQQTRGLL
1827	TAYSQQTRGLLG
1829	SMSYTWGALITP
1838	SPGALVVGVI
1843	LPRRGPRRL

Results of the secondary screening with individual peptides are summarized in **Table 5**. Altogether ~20% of subjects (G05, G18, H02, H03, H04, H10, H12, H19, H32, H38) showed a significant IFN-gamma T-cell response against one or more of the peptides. In some cases the observed number of ELISPOTs was clearly elevated, but not statistically significant above background. In these cases, PBMC (donors H03, H10, H33, H38) were stimulated with the respective peptides in vitro (2 rounds of in vitro priming, see Material & Methods) in order to increase the peptide specific response. Several peptides were confirmed in this way, results are again summarized in **Table 5**.

Peptides A3-A7 represent overlapping 15mers spanning the sequence TNPKPQRKTKRNTNRRPQD. Since they all react with PBMC from donor H03, the minimal sequence of the epitope is located within the sequence PQRKTKRNTNR. Prediction algorithms indicate that QRKTKRNTN and QRKTKRNT represent ligands of HLA-B*08, whereas RKTNRNTNR most probably binds to HLA-B*2705.

Peptides C64-C70 represent overlapping 15mers spanning the sequence KGGRKPARLIVFPDLGVRVCE. C64 and C70 react with PBMC from donor H32 and H38, respectively. The minimal sequence of the epitope is therefore located within the sequence ARLIVFPDL. Prediction algorithms indicate that ARLIVFPDL represents a ligand of HLA-B*2705 and HLA-B*2709.

Table 5. Summary of HCV peptides reactive with PBMC.

Numbers represent peptide-specific IFN-gamma secreting T-cells/ 10^6 PBMC calculated from ELISPOT results (duplicate determinations); values > 8 (>3x over background) were regarded statistically significant. Donors H32 and H33 are spontaneously recovered patients.

[illegible]

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C118				35									
C120				25	45					105			
C134					20								
C138												30	

	Donors reactive directly ex vivo in IFN-gamma ELISpot with HCV-derived peptides										...reactive after 2 rounds of in vitro stimulation		
Peptide ID	G05	G18	H02	H03	H04	H10	H12	H19	H32 SPR	H38	H03	H10	H33 SPR
1557				20							75		
1577			15		30								165
1579			45		35						75		
1605					25								
1615				30							325		
1624	30		55		30			420					
1628			40		45		25			20			
1629			30		70		15						
1798					25							115	
1799							20					90	
1800										35		95	
1801						20				20			
A3											80		
A4				15									
A5											110		
A7											70		
A78					25								
A170					35								
A212					60								
A241					35								
B08					55								
B38									30				
B76					35								
C64									30				
C70										20			
C92					25								
C94					25								
C97					35								
C98					70								
C100		60			70								

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Example V. Binding of HCV derived peptides to HLA class II molecules

In addition to the peptides listed in Table 1, several new peptides incorporating sequences from overlapping reactive peptides or avoiding critical residues like cysteine were synthesized (Table 4). These were retested for their affinities to class II soluble HLA molecules, and results were compared to those obtained with the original (Table 6).

Table 6. Binding of selected HCV-derived peptides and their 15-mer counterparts to soluble HLA class II molecules ("+++" strong affinity, "++" intermediate affinity, "+" weak affinity, "-" no affinity, "nd" not done; core binding motifs are underlined).

Peptide ID	Peptide sequences	Binding to soluble HLA-DRB1*				
		0101	0401	0404	0701	1101
1798	IG <u>LGKVLVDILAGY</u> GAGVAGALVAFK	-	-	+	++	+/-
B84	GSIG <u>LGKVLVDILAG</u>	+	+	+		-
B86	IG <u>LGKVLVDILAGY</u> G	+	++	+	+	+/-
B88	<u>LGKVLVDILAGY</u> GAG	+	++	+		
B92	LVDILAGYGAGVAGA	+	-			
B94	DILAGY <u>GAGVAGALV</u>	+	-	-	-	
B96	LAGY <u>GAGVAGALVAF</u>	++	++	-	+/-	+/-
1799	AAWYELTPAETTVRLR	+++	+	+	-	+/-
B46	AGAAWYELTPAETTV	+++	+++	+++	-	+/-
B48	AAWYELTPAETTVRL	+++	+++	+++	-	+/-
1827	TAYSQOTRGLLG	++	-	+/-	+	+
C114	TAYSQOTRGLLGCIIV	+++	+/-	+/-	+	++
1829	SMSYTW <u>TGALITP</u>	+	-	-	+	+/-
1604	VVCCSMSYTW <u>TGALITP</u> C	+	+	++	++	+
1650	VDYPYRLWHYPCTVNF <u>TIFKVRMYVGGVEHRL</u>					
A130	DYPYRLWHYPCTVNF	+	++	+/-		
A131	YPYRLWHYPCTVNF		-			
A135	<u>LWHYPCTVNF</u> TIFKV	-	-		++	

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A141	TVNFTIFKVRMYVGG	-	-	+/ -	++	
A145	TIFKVRMYVGGVEHR	+/ -	-			
1651	VDYPYRLWHYPCTVNYTIFKIRMYVGGVEHRL					
1800	DYPYRLWHYPCTVNYTIFKI	-	-	+/ -	++	-
A147	DYPYRLWHYPCTVNY	-	-			
A152	LWHYPCTVNYTIFKI	-	-			
A158	TVNYTIFKIRMYVGG	-	-	+/ -		
A162	TIFKIRMYVGGVEHR	+/ -	-			
1817	RMYVGGVEHRL	-	-	+/ -		
1426	HMWNFISGIQYLAGLSTLPGNPA	+	+	++	++	+
1425	NFISGIQYLAGLSTLPGNPA	++	++	++	nd	nd
1006	MWNFISGIQYLAGLSTLPGN	++	+	++	nd	nd

Abolished affinities to DRB1*0101 and DRB1*0401 molecules in the case of peptide 1798 in comparison with its shorter counterparts (B84 - B96) is probably due to the long sequence (26 amino acids) which can have a secondary structure that prevents binding. It is to be expected that in vivo, upon proteolytic cleavage, peptide 1798 will give rise to two shorter class II epitopes. Removed cystein (C) residues in peptides 1827 and 1829 (derivatives of peptides C114 and 1604, respectively) seem to be crucial for binding to DRB1*0401 molecules but do not essentially change affinities to other tested DR subtypes.

Example VI. Identification and Characterization of HCV-epitope hotspots

Here, a T-cell epitope hotspot (thereafter referred to as "hotspot") is defined as a short peptide sequence at least comprising more than one T-cell epitope. For example, two or more epitopes may be located shortly after each other (shortly being defined as less than 5-10 amino acids), or directly after each other, or partially or even fully over-lapping. Hotspots may contain only class I or class II epitopes, or a combination of both. Epitopes in hotspots may have different HLA restrictions.

Due to the highly complex and selective pathways of class I and class II antigen processing, referred to in the introduction, T-cell epitopes cannot be easily predicted within the sequence of a polypeptide. Though widely used, computer algorithms for T-cell epitope prediction have a high rate of both false-negatives and false-positives.

Thus, as even individual T-cell epitopes are not obvious within the sequence of a polypeptide, the same is even more the case for hotspots. Several radically different experimental approaches are combined according to the present invention for T-cell epitope identification, including epitope capture, HLA-transgenic animals and *in vitro* stimulation of human mononuclear cells. All three approaches are systematically applied on overlapping peptides spanning the antigen of interest, enabling comprehensive identification of epitopes (refer to CMV Epitope Capture patent). Upon such a comprehensive analysis, not limited to a particular HLA allele, but rather unravelling all possibly targeted epitopes within a population, epitope hotspots may become apparent. Within an antigen, only few if any sequences show characteristics of hotspots. Thus the identification of a hotspot is always a surprising event:

T-cell epitope hotspots offer important advantages: Hotspots can activate and can be recognized by different T-cell clones of a subject. Hotspots (when comprising epitopes with different HLA restriction) can interact with T-cells from different non HLA-matched individuals.

Epitope-based vaccines, so far have aimed at selected prevalent HLA-alleles, for instance HLA-A2, which is expressed in about half of Caucasians. Since other alleles are less frequent, epitope-based vaccines with broad worldwide population coverage will have to comprise many different epitopes. The number of chemical entities (for instance peptides) of a vaccine is limited by constraints originating from manufacturing, formulation and product stability.

Hotspots enable such epitope-based vaccines with broad worldwide population coverage, as they provide a potentially high number

of epitopes by a limited number of peptides.

Table 7: T-cell epitope hotspots in conserved regions of HCV.

Hotspots (incl. some variations) are shown in bold, epitopes contained within the hotspots in normal font. Peptide number and sequence, as well as HLA-class I and class II coverage are given. Source data refers to Examples and Tables within this specification, or literature references.

peptide				
ID	peptide sequence	class I	class II	source data
1835	KFPGGGQIVGGVYLLPRRGPRLGVRATRK	A2, A3, B7	DR11	Example III, VI
83	KFPGGGQIVGGVYLLPRRGPRL	A2 B7	DR11	Example VI
1051	YLLPRRGPRL	A2		Bategay 1995
1843	LPRRGPRL	B7		Example III
	GPRLGVRAT	B7		Koziel 1993
	RLGVRATRK	A3		Chang 1999
84	GYKVLVLNPSVAAT		DR1,4,7,11	Tab.2:A200-A206
	AYAAQGYKVL	A24		prediction
84EX	AYAAQGYKVLVLNPSVAAT	A24	DR1,4,7,11	Example VI
87	DLMGYIPAV	A2		Sarobe 1998
	GYIPLVGAPL	A24		prediction
87EX	DLMGYIPLVGAPL	A2,A24		Example VI
89	CINGVCWTV	A2		Koziel 1995
1577	GEVQVVSTATQSFLAT		DR 4, 7	Tab.2
89EX	GEVQVVSTATQSFLATCINGVCWTV	A2	DR 4, 7	Example VI
1426	HMWNFISGIQYLAGLSTLPGNPA	A2	DR1,4,7,11	Example VII
1006	MWNFISGIQYLAGLSTLPGN			Example VII
1425	NFISGIQYLAGLSTLPGNPA			Example VII
	QYLAGLSTL	A24		prediction
1334	HMWNFISGI	A2		Wentworth 1996
1650	VDYPYRLWHYPCTVNFTIFKVRMYVGGVEHRL	Cw7,A2,A24, A11,A3	DR1,4,7,11	Tab. 2,3,6 Example III
1836	DYPYRLWHYPCTVNFTIFKI	Cw7,A2,A24, A11	DR1,4,7,11	Tab. 2,3,6
1846	DYPYRLWHYPCTVNFTIFKV	Cw7,A2,A24, A11	DR1,4,7,11	Tab. 2,3,6 Example III
1651	VDYPYRLWHYPCTVNFTIFKIRMYVGGVEHRL			Tab. 2,3,6
1800	DYPYRLWHYPCTVNFTIFKI	Cw7,A24,A11	DR7	Tab. 2,5,6
1754	DYPYRLWHY	Cw7		Lauer 2002
1815	TVNYTIFKI	A11		prediction

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1816	TINYTIFK	A11		Koziel 1995
	TVNFTIFKV	A11		prediction
	HYPCTVNYTI	A24		prediction
	HYPCTVNFTI	A24		prediction
	RMVVGVEHR	A3		Chang 1999
1799	AAWYELTPAETTVRLR	B7? B35	DR1, 4	Tab. 2,5,6
1818	TPAETTVRL	B7? B35		Ibe 1998
1827EX	GWRL LAPITAYSQQTRGLLGCIV	A2,A3,A24,	DR1,4,7,11	Example VI
		B8		
C114	TAYSQQTRGLLGCIV	A24, B8?	DR1,4,7,11	Tab.2, 6
1827	TAYSQQTRGLLG	A24, B8	DR1, 7,11	Tab.6
C112	GQGWRL LAPITAYSQ	A3?,A2?,	DR1	Tab.2, 5
	RLLAPITAY	A3		prediction
C114EX	GQGWRL LAPITAYSQQTRGLLGCIV	A24,A3?,A2?,	DR1,4,7,11	Tab. 2, 5, 6
		B8?		
		A24,A3?,A2?,	DR1, 7,11	Tab. 2, 5, 6
1827EX	GQGWRL LAPITAYSQQTRGLLG	B8?		
1801	AYSQQTRGLL	A24		Tab. 5
1819	AYSQQTRGL	A24		Kurokohchi 2001
1798	IGLGKVLVDILAGYGAGVAGALVAFK	A2,24,3,11	DR1,4,7	Tab.2,3,5,6
1820	ILAGYGAGV	A2		Bategay 1995
1821	VAGALVAFK	A3,11		Chang 1999
	GYGAGVAGAL	A24		prediction
1604	VVCCSMSYTWGALITPC	A2,A24,B7	DR1,4,7,11	Tab.2,3,6
1829	SMSYTWGALITP	A2,A24,B7,	DR1, 7,11	Tab.6
	SMSYTWGAL	A2,B7		prediction
	SYTWGALI	A24		prediction
1579	FTDNSSPPAVPQTFQV	A1,2;B7,51	DR53=B4*01	Tab.5
1624	LED RDRSELSPLLLSTTEW	A1,2,3,26	DR7	Tab.2,3,5
		B8,27,4402,60		
1848	LED RDRSELSPLLLST	A1,2,3,26,	DR7	Example VI
		B8,27,4402,60		
	RSELSPLLL	A1		prediction
	ELSPLLLST	A2,A3		prediction
	DRDRSEL SPL	A26,B27		prediction
	LED RDRSEL	B08,B4402		prediction
1824	LED RDRSEL	B60		Wong 2001
1547	YLVAYQATVCARAQAPPSWD	A2	DR1,4,7,11	Tab.2,3
1822	YLVAYQATV	A2		Wentworth 1996
A1A7	MSTNPKPQRKTKRNTNR	A11,B08,B27		Tab.5

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A3A7	PQRKTKRNTNR	B08, B27	Tab.5
	QRKTKRNTN	B08	prediction
	RKTKRNTNR	B2705	prediction
	MSTNPKPQR	A11	prediction
	MSTNPKPQK	A11	Wong 1998
A122EX	LINTNGSWHINRTALNCNDSL	A2, 2, 3, B8	DR1, 4, 7, 11 Tab.2, 3
A122	NGSWHINRTALNCNDSL	A2	DR1, 4, 7, 11 Tab.2, 3
	LINTNGSWHI	A2, 3	prediction
	RTALNCNDSL	A2	prediction
1825	LINTNGSWHINRTALN	A2, 3, B8	prediction
1826	SWHINRTALN	B8	prediction
A241	TTILGIGTVLDQAET	A2, A3	DR1, 4 Tab.2, 5
	TTILGIGTV	A2	prediction
	TILGIGTVL	A3	prediction
B8B38	FDSSVLCECYDAGAAWYE	A1, 2, 3, 26	Tab.5
B8	FDSSVLCECYDAGCA	A3, A26	Tab.5
	VLCECYDAGA	A2	prediction
B38	VVLCECYDAGAAWYE	A1	Tab.5
C70EX	ARLIVFPDLGVRVCEKMALY	A2, A3, B27	Tab.5
C64-C70	ARLIVFPDL	B*2705?, *2709?	Tab.5
1831	RLIVFPDLGV	A2	Gruener 2000
1832	RVCEKMALY	A3	Wong 1998
C92	AFCSAMYVGDLCGSV	A2, B51	DR1, 4 Tab.2, 5
C97	GVLFGLAYFSMVGWV	A2, 3, 26, B2705, 51	DR1, 4, 7 Tab.5
C106	TRVPYFVRAQGLIRA	A3, 24, B7, B8, B2705	DR1, 4, 7 Tab.2, 5
C134	TTLLFNILGGWVAAQ	A2	DR1, 7, 11 Tab.2, 5
1823	LLFNILGGWV	A2	Bategay 1995

Example VII. HCV epitope hotspot Ipep 1426 contains at least HLA-A*0201 and several promiscuous class II T-cell epitopes

The major objective of this experiment was to compare the immunogenicity of the "hotspot" Ipep 1426, which contains at least one HLA-A*0201 epitope (Ipep 1334) and 2 promiscuous class II epitopes (Ipeps 1006 and 1425), to the individual epitopes. To this end peripheral blood mononuclear cells (PBMC) from several healthy HLA-typed blood donors were stimulated in vitro either with 1426 or a mixture of 1334, 1006, 1425. Three rounds of

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stimulation were performed resulting in oligoclonal T cell lines. Then, responses against all four peptides were assessed by interferon-gamma (IFN- γ) ELISpot analysis.

Peptide 1426, induces T cell responses similarly well as individual epitopes comprised within its sequence. In particular, CD8 positive T cells directed against the HLA-A*0201 restricted epitope 1334 were successfully generated.

Table 8: peptide induced IFN- γ secretion of oligoclonal T cell lines. Lines were generated from two HLA-typed healthy individuals by 3 rounds of in vitro priming with either peptide 1426 or a mixture of peptides 1006+1425+1334. The reactivity of CD4 and CD8 positive T cells in these lines was assessed by IFN- γ ELISpot ("+++" very strong, "++" strong, "+" significant, "-" no IFN-gamma secretion).

Donor HLA	A*0201, A*03, B7, B60; DRB1*1501, -B1*1302		A*0206, A*01, B27, B50; DRB1*0401, -B1*1402	
Peptide ID	line raised against 1426	line raised against 1006+1425+1334 4	line raised against 1426	line raised against 1006+1425+1334 4
1006	++	++	++	++
1425	+++	+++	+++	++
1334	+	+	-	-
1006+1425+1334	++	++	++	++
1426	+++	+++	+++	++
84 (HCV derived negative control)	-	-	-	-

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Claims:

1. Method for isolating Hepatitis C Virus peptides (HPs) which have a binding capacity to a MHC/HLA molecule or a complex comprising said HCV-peptide and said MHC/HLA molecule characterized by the following steps:

- providing a pool of HCV-peptide, said pool containing HCV-peptides which bind to said MHC/HLA molecule and HCV-peptides which do not bind to said MHC/HLA molecule,
- contacting said MHC/HLA molecule with said pool of HCV-peptides whereby a HCV-peptide which has a binding capacity to said MHC/HLA molecule binds to said MHC/HLA molecule and a complex comprising said HCV-peptide and said MHC/HLA molecule is formed,
- detecting and optionally separating said complex from the HCV-peptides which do not bind to said MHC/HLA molecule and
- optionally isolating and characterising the HCV-peptide from said complex.

2. Method for isolating HCV T cell epitopes which have a binding capacity to a MHC/HLA molecule or a complex comprising said epitope and said MHC/HLA molecule characterized by the following steps:

- providing a pool of HCV-peptides, said pool containing HCV-peptides which bind to a MHC/HLA molecule and HCV-peptides which do not bind to said MHC/HLA molecule,
- contacting said MHC/HLA molecule with said pool of HCV-peptides whereby a HCV-peptide which has a binding capacity to said MHC/HLA molecule binds to said MHC/HLA molecule and a complex comprising said HCV-peptide and said MHC/HLA molecule is formed,
- detecting and optionally separating said complex from the HCV-peptides which do not bind to said MHC/HLA molecule,
- optionally isolating and characterising the HCV-peptide from said complex,
- assaying said optionally isolated HCV-peptide or said complex in a T cell assay for T cell activation capacity and
- providing the optionally isolated HCV-peptide with a T cell activation capacity as HCV T cell epitope or as complex.

3. Method according to claim 1 or 2, characterized in that said pool of HCV-peptides is selected from the group consisting of a pool of peptides, especially overlapping peptides, a pool of protein fragments, a pool of modified peptides, a pool obtained from antigen-presenting cells, preferably in the form of total lysates or fractions thereof, especially fractions eluted from the surface or the MHC/HLA molecules of these cells, a pool comprised of fragments of cells, especially HCV-containing cells, tumor cells or tissues, especially from liver, a pool comprised of peptide libraries, pools of (poly)-peptides generated from recombinant DNA libraries, especially derived from pathogens or tumor cells, a pool of proteins and/or protein fragments from HCV or mixtures thereof.

4. Method according to any one of claims 1 to 3, characterized in that said MHC/HLA molecules are selected from HLA class I molecules, HLA class II molecules, non classical MHC/HLA and MHC/HLA-like molecules or mixtures thereof, or mixtures thereof.

5. Method according to any one of claims 1 to 4, characterized in that said characterising of the HCV-peptides of the complex is performed by using a method selected from the group consisting of mass spectroscopy, polypeptide sequencing, binding assays, especially SDS-stability assays, identification of HCV-peptides by determination of their retention factors by chromatography, especially HPLC, or other spectroscopic techniques, especially violet (UV), infra-red (IR), nuclear magnetic resonance (NMR), circular dichroism (CD) or electron spin resonance (ESR), or combinations thereof.

6. Method according to any one of claims 1 to 5, characterized in that it is combined with a cytokine secretion assay, preferably with an Elispot assay, an intracellular cytokine staining, FACS or an ELISA.

7. Method according to any one of claims 1 to 6, characterized in that said T cell assay comprises the mixing and incubation of said complex with isolated T cells and subsequent measuring cytokine secretion or proliferation of said isolated T cells.

8. Method according to any one of claims 1 to 6, characterized in that said T cell assay comprises measuring up-regulation of activation markers, especially CD69, CD38, or down-regulation of surface markers, especially CD3, CD8 or TCR.

9. Method according to any one of claims 1 to 8, characterized in that said T cell assay comprises measuring up-/down-regulation of mRNAs involved in T cell activation, especially by real-time RT-PCR.

10. Method according to any one of claims 1 to 8, characterized in that said T cell assay is selected from T cell assays measuring phosphorylation/de-phosphorylation of components downstream of the T cell receptor, especially p56 lck, ITAMS of the TCR and the zeta chain, ZAP70, LAT, SLP-76, fyn, and lyn, T cell assays measuring intracellular Ca⁺⁺ concentration or activation of Ca⁺⁺-dependent proteins, T cell assays measuring formation of immunological synapses, T cell assays measuring release of effector molecules, especially perforin, granzymes or granulolysin or combinations of such T cell assays.

11. T cell epitopes identifiable by a method according to any one of claims 2 to 10, said T cell epitopes being selected from the group consisting of polypeptides A120-A124, B25-B30, B46-B48, B84-B92, C106, C113-C114, 1627, 1628, 1629, 1604, 1630, C97, 1547, B94-B98, A272-A276, B120, B122, C108, C134, C152, 1606, 1607, 1577, 1578, B50-52, 1623, C130, 1603, C96, C191, A216-A224, A242-A244, C92-C93, A174, B32-B38, B100-B102, C135, C162, 1618, 1622, 1624, 1546, 1556, A114, B58, B112-B118, B18-B22, C112, C116, C122, C127, C144, C159-C160, C174, 1558, 1581, C95, C129, C157-C158, A254-A258, 1605, C109, C161, 1547, 1555, 1558, 1559, 1560, 1563, 1592, 1605, 1616, 1621, 1623, 1625, 1649, 1650, 1651, 1652, 1654, 1655, 1656, 1545, 1552, 1557, 1615, 1617, 1631, 1632, 1641, 1647, 1653, A141, C114, C134, C135 and 1426.

12. HLA A0201 binding epitopes with T cell activating capacity identifiable by a method according to any one of claims 2 to 10 using HLA A0201 molecules as MHC/HLA molecules, said HLA A0201

binding epitopes being selected from the group consisting of polypeptides 1545, 1552, 1555, 1558, 1559, 1560, 1577, 1592, 1604, 1605, 1615, 1617, 1621, 1627, 1631, 1632, 1641, 1647, 1650, 1651, 1652, 1653, 1654, 1655 as specified in Table 1.

13. HLA-B*0702 binding epitopes with T cell activating capacity identifiable by a method according to any one of claims 2 to 10 using HLA-B*0702 molecules as MHC/HLA molecules, said HLA B*0702 binding epitopes being selected from the group consisting of polypeptides 1506, 1526, 1547, 1552, 1553, 1555, 1558, 1562, 1563, 1565, 1577, 1578, 1580, 1587, 1592, 1604, 1605, 1621, 1623, 1624, 1627, 1628, 1647, 1650, 1651, 1843 with sequence LPRRGPRRL (contained in 1506) and 1838 with sequence SPGALVVGVI (contained in 1587) as minimal HLA-B*0702 epitopes.

14. Epitope or peptide according to any one of claims 11 to 13 characterized in that it further comprises 1 to 30, preferably 2 to 10, especially 2 to 6, naturally occurring amino acid residues, especially at the N-terminus, the C-terminus or at the N- and C-terminus.

15. Epitope or peptide according to any one of claims 11 to 14, characterized in that it further comprises a non-naturally occurring amino acid(s), preferably 1 to 1000, more preferred 2 to 100, especially 2 to 20 non-naturally occurring amino acid residues, at the N-terminus, the C-terminus or at the N- and C-terminus.

16. Use of an epitope or peptide according to any one of claims 11 to 14 for the preparation of a vaccine, especially of a HLA restricted vaccine, for treating or preventing hepatitis C virus (HCV) infections.

17. Vaccine for treating or preventing hepatitis C virus (HCV) infections comprising an epitope according to any one of claims 11 to 15.

18. HLA specific vaccine for treating or preventing hepatitis C virus (HCV) infections comprising an epitope or peptide according to any one of claims 11 to 15.

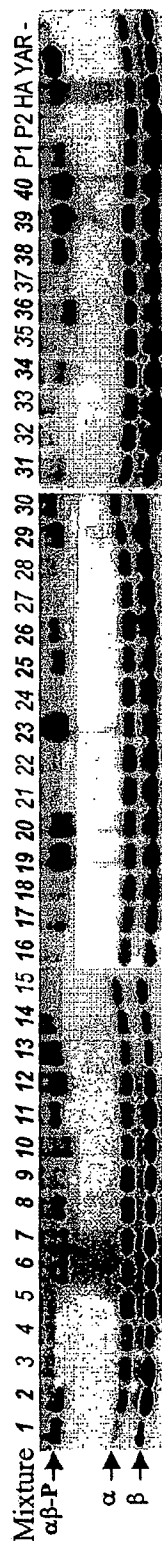
19. Vaccine as defined in any one of claims 16 to 18, characterized in that it further comprises an immunomodulating substance, preferably selected from the group consisting of polycationic substances, especially polycationic polypeptides, immunomodulating nucleic acids, especially deoxyinosine and/or deoxyuracile containing oligodeoxynucleotides, or mixtures thereof.
20. Vaccine as defined in any one of claims 16 to 19, characterized in that it further comprises a pharmaceutically acceptable carrier.
21. Vaccine as defined in any one of claims 16 to 20, characterized in that said epitope is provided in a form selected from peptides, peptide analogues, proteins, naked DNA, RNA, viral vectors, virus-like particles, recombinant/chimeric viruses, recombinant bacteria or dendritic cells pulsed with protein/peptide/RNA or transfected with DNA comprising the epitopes.
22. T cells, a T cell clone or a T cell population or preparation specifically recognizing an epitope or peptide according to any one of claims 11 to 15.
23. Use of T cells, a T cell clone or a T cell population or preparation according to claim 22 for identification of heteroclitic epitopes.
24. Use of T cells, a T cell clone or a T cell population or preparation according to claim 22 for the preparation of a composition for therapy of HCV patients.
25. Use of the peptides with formulae QRKTKRNTN or QRKTKRNT, or 1615, 1616, 1617 in particular 9meric peptides derived from the latter 3 peptides with formulae SAKSKFGYG, SAKSKYGYG, or SARSKYGYG as HLA-B*08 epitopes, especially for the preparation of a pharmaceutical preparation for a HLA-B*08 specific vaccine.
26. Use of the peptides with the formulae RKTKRNTNR as HLA-B*2705 epitope, especially for the preparation of a pharmaceut-

ical preparation for a HLA-B*2705 specific vaccine.

27. Use of the peptides with the formulae ARLIVFPDL as HLA-B*2705 and HLA-B*2709 specific vaccine.

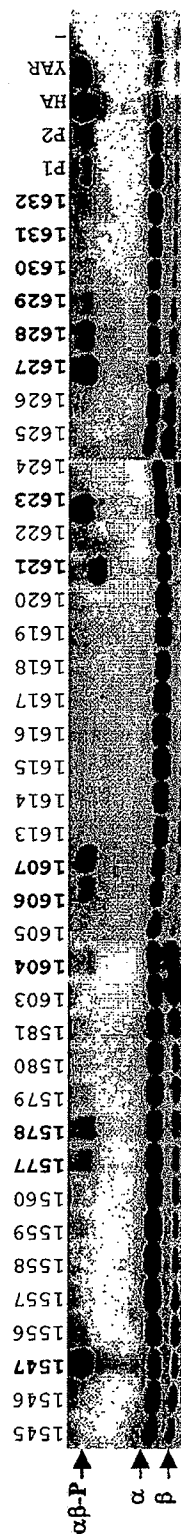
28. Use of peptides as specified in Tab. 7, said peptides representing T-cell epitope hotspot and selected from the group of peptides 1835, 84EX, 87EX, 89EX, 1426, 1650, 1836, 1846, 1651, 1800, 1799, C114, 1827, C112, C114EX, 1827EX, 1798, 1604, 1829, 1579, 1624, 1848, 1547, A1A7, A122EX, A122, 1825, A241, B8B38, C70EX, C92, C97, C106, and C134.

Figure 2. Peptide pools that bind to DRB1*0401



	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
M21	A2	A4	A6	A8	A10	A12	A14	A16	A18	A20	A22	A24	A26	A28	A30	A32	A34	A36	A38	A40
M22	A38	A40	A42	A44	A46	A48	A50	A52	A54	A56	A58	A60	A62	A64	A66	A68	A70	A72	A74	A76
M23	A74	A76	A78	A80	A82	A84	A86	A88	A90	A92	A94	A96	A98	A100	A102	A104	A106	A108	A110	A112
M24	A110	A112	A114	A116	A118	A120	A122	A124	A126	A128	A130	A132	A134	A136	A138	A140	A142	A144	A146	A148
M25	A146	A148	A150	A152	A154	A156	A158	A160	A162	A164	A166	A168	A170	A172	A174	A176	A178	A180	A182	A184
M26	A182	A184	A186	A188	A190	A192	A194	A196	A198	A200	A202	A204	A206	A208	A210	A212	A214	A216	A218	A220
M27	A218	A220	A222	A224	A226	A228	A230	A232	A234	A236	A238	A240	A242	A244	A246	A248	A250	A252	A254	A256
M28	A254	A256	A258	A260	A262	A264	A266	A268	A270	A272	A274	A276	B2	B4	B6	B8	B10	B12	B14	B16
M29	B14	B16	B18	B20	B22	B24	B26	B28	B30	B32	B34	B36	B38	B40	B42	B44	B46	B48	B50	B52
M30	B50	B52	B54	B56	B58	B60	B62	B64	B66	B68	B70	B72	B74	B76	B78	B80	B82	B84	B86	B88
M31	B86	B88	B90	B92	B94	B96	B98	B100	B102	B104	B106	B108	B110	B112	B114	B116	B118	B120	B122	B124
M32	C2	C4	C6	C8	C10	C12	C14	C16	C18	C20	C22	C24	C26	C28	C30	C32	C34	C36	C38	C40
M33	C38	C40	C42	C44	C46	C48	C50	C52	C54	C56	C58	C60	C62	C64	C66	C68	C70	C72	C74	C76
M34	C74	C76	C78	C80	C82	C84	C86	C88	C90	C92	C94	C96	C98	C100	C102	C104	C106	C108	C110	C112
M35	C102	C104	C106	C108	C110	C112	C114	C116	C118	C120	C122	C124	C126	C128	C130	C132	C134	C136	C138	C140
M36	C121	C122	C123	C124	C125	C126	C127	C128	C129	C130	C131	C132	C133	C134	C135	C136	C137	C138	C139	C140
M37	C139	C140	C141	C142	C143	C144	C145	C146	C147	C148	C149	C150	C151	C152	C153	C154	C155	C156	C157	C158
M38	C157	C158	C159	C160	C161	C162	C163	C164	C165	C166	C167	C168	C169	C170	C171	C172	C173	C174	C175	C176
M39	C175	C176	C177	C178	C179	C180	C181	C182	C183	C184	C185	C186	C187	C188	C189	C190	C191	C192	C193	C194
M40	C192	C193	C194	C195	C196	C197	C198	C199	C200	C201	C202	C203	C204	C205	C206	C207	C208	C209	C210	C211

Figure 4. Individual peptides that bind to DRB1*0401



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M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
M21	A2	A4	A6	A8	A10	A12	A14	A16	A18	A20	A22	A24	A26	A28	A30	A32	A34	A36	1631 1632
M22	A38	A40	A42	A44	A46	A48	A50	A52	A54	A56	A58	A60	A62	A64	A66	A68	A70	A72	1624 1625
M23	A74	A76	A78	A80	A82	A84	A86	A88	A90	A92*	A94	A96	A98	A100	A102	A104	A106	A108	1577 1578
M24	A110	A112	A114	A116	A118	A120	A122	A124	A126	A128	A130	A132	A134	A136	A138	A140	A142	A144	1579 1580
M25	A146	A148	A150	A152	A154	A156	A158	A160	A162	A164	A166	A168	A170	A172	A174	A176	A178	A180	1547 1623
M26	A182	A184	A186	A188	A190	A192	A194	A196	A198	A200	A202	A204	A206	A208	A210	A212	A214	A216	1606 1607
M27	A218	A220	A222	A224	A226	A228	A230	A232	A234	A236	A238	A240	A242	A244	A246	A248	A250	A252	1626 1613
M28	A254	A256	A258	A260	A262	A264	A266*	A268	A270	A272	A274	A276	B2	B4	B6	B8	B10	B12	1604 1605
M29	B14	B16	B18	B20	B22	B24	B26	B28	B30	B32*	B34	B36	B38	B40	B42	B44	B46	B48	1618 1619
M30	B50	B52	B54	B56	B58	B60	B62	B64	B66	B68	B70	B72*	B74	B76	B78	B80	B82	B84	1559 1560
M31	B86	B88	B90	B92	B94	B96	B98	B100	B102	B104	B106	B108	B110	B112	B114	B116	B118	B120	B124
M32	C2	C4	C6	C8	C10	C12	C14	C16	C18	C20	C22	C24	C26	C28	C30	C32	C34	C36	1614 1615
M33	C38	C40	C42	C44	C46	C48	C50	C52	C54	C56	C58	C60	C62	C64	C66	C68	C70	C72	1616 1617
M34	C74	C76	C78	C80	C82	C84	C86	C88	C90	C92	C94	C96	C98	C100	C102	C104	C106	C108	C110
M35	C102	C103	C104	C105	C106	C107	C108	C109	C110	C111	C112	C113	C114	C115	C116	C117	C118	C119	C120
M36	C121	C122	C123	C124	C125	C126	C127	C128	C129	C130	C131	C132	C133	C134	C135	C136	C137	C138	1620 1621
M37	C139	C140	C141	C142	C143	C144	C145	C146	C147	C148	C149	C150	C151	C152	C153	C154	C155	C156	PBS
M38	C157	C158	C159	C160	C161	C162	C163	C164	C165	C166	C167	C168	C169	C170	C171	C172	C173	C174	PBS
M39	C175	C176	C177	C178	C179	C180	C181	C182	C183	C184	C185	C186	C187	C188	C189	C190	C191	C192	PBS
M40	1629	1628	1630	1545	1581	1546	1622	1603	1557	1558	1627	PBS	PBS	PBS	PBS	C193	C195	C197	PBS

Figure 6. Individual peptides that bind to DRB1*0101



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	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
M21	A2	A4	A6	A8	A10	A12	A14	A16	A18	A20	A22	A24	A26	A28	A30	A32	A34	A36	1631	1632
M22	A38	A40	A42	A44	A46	A48	A50	A52	A54	A56	A58	A60	A62	A64	A66	A68	A70	A72	1624	1625
M23	A74	A76	A78	A80	A82	A84	A86	A88	A90	A92*	A94	A96	A98	A100	A102	A104	A106	A108	1577	1578
M24	A110	A112	A114	A116	A118	A120	A122	A124	A126	A128	A130	A132	A134	A136	A138	A140	A142	A144	1579	1580
M25	A146	A148	A150	A152	A154	A156	A158	A160	A162	A164	A166	A168	A170	A172	A174	A176	A178	A180	1547	1623
M26	A182	A184	A186	A188	A190	A192	A194	A196	A198	A200	A202	A204	A206	A208	A210	A212	A214	A216	1606	1607
M27	A218	A220	A222	A224	A226	A228	A230	A232	A234	A236	A238	A240	A242	A244	A246	A248	A250	A252	1626	1613
M28	A254	A256	A258	A260	A262	A264	A266*	A268	A270	A272	A274	A276	B2	B4	B6	B8	B10	B12	1604	1605
M29	B14	B16	B18	B20	B22	B24	B26	B28	B30	B32*	B34	B36	B38	B40	B42	B44	B46	B48	1618	1619
M30	B50	B52	B54	B56	B58	B60	B62	B64	B66	B68	B70	B72*	B74	B76	B78	B80	B82	B84	1559	1520
M31	B86	B88	B90	B92	B94	B96	B98	B100	B102	B104	B106	B108	B110	B112	B114	B116	B118	B120	1622	1624
M32	C2	C4	C6	C8	C10	C12	C14	C16	C18	C20	C22	C24	C26	C28	C30	C32	C34	C36	1614	1615
M33	C38	C40	C42	C44	C46	C48	C50	C52	C54	C56	C58	C60	C62	C64	C66	C68	C70	C72	1616	1617
M34	C74	C76	C78	C80	C82	C84	C86	C88	C90	C92	C94	C96	C98	C100	C102	C104	C106	C108	1601	1602
M35	C102	C103	C104	C105	C106	C107	C108	C109	C110	C111	C112	C113	C114	C115	C116	C117	C118	C119	1620	1621
M36	C121	C122	C123	C124	C125	C126	C127	C128	C129	C130	C131	C132	C133	C134	C135	C136	C137	C138	1620	1621
M37	C139	C140	C141	C142	C143	C144	C145	C146	C147	C148	C149	C150	C151	C152	C153	C154	C155	C156	PBS	PBS
M38	C157	C158	C159	C160	C161	C162	C163	C164	C165	C166	C167	C168	C169	C170	C171	C172	C173	C174	PBS	PBS
M39	C175	C176	C177	C178	C179	C180	C181	C182	C183	C184	C185	C186	C187	C188	C189	C190	C191	C192	PBS	PBS
M40	1629	1628	1630	1545	1581	1546	1622	1603	1557	1558	1627	PBS	PBS	PBS	PBS	C193	C195	C197	PBS	PBS

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Figure 7. Individual peptides that bind to DRB1*0701

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
M21	A2	A4	A6	A8	A10	A12	A14	A16	A18	A20	A22	A24	A26	A28	A30	A32	A34	A36	1631	1632
M22	A38	A40	A42	A44	A46	A48	A50	A52	A54	A56	A58	A60	A62	A64	A66	A68	A70	A72	1624	1625
M23	A74	A76	A78	A80	A82	A84	A86	A88	A90	A92*	A94	A96	A98	A100	A102	A104	A106	A108	1577	1578
M24	A110	A112	A114	A116	A118	A120	A122	A124	A126	A128	A130	A132	A134	A136	A138	A140	A142	A144	1579	1580
M25	A146	A148	A150	A152	A154	A156	A158	A160	A162	A164	A166	A168	A170	A172	A174	A176	A178	A180	1547	1623
M26	A182	A184	A186	A188	A190	A192	A194	A196	A198	A200	A202	A204	A206	A208	A210	A212	A214	A216	1606	1607
M27	A218	A220	A222	A224	A226	A228	A230	A232	A234	A236	A238	A240	A242	A244	A246	A248	A250	A252	1626	1613
M28	A254	A256	A258	A260	A262	A264	A266*	A268	A270	A272	A274	A276	B2	B4	B6	B8	B10	B12	1604	1605
M29	B14	B16	B18	B20	B22	B24	B26	B28	B30	B32*	B34	B36	B38	B40	B42	B44	B46	B48	1618	1619
M30	B50	B52	B54	B56	B58	B60	B62	B64	B66	B68	B70	B72*	B74	B76	B78	B80	B82	B84	1559	1560
M31	B86	B88	B90	B92	B94	B96	B98	B100	B102	B104	B106	B108	B110	B112	B114	B116	B118	B120	B122	B124
M32	C2	C4	C6	C8	C10	C12	C14	C16	C18	C20	C22	C24	C26	C28	C30	C32	C34	C36	1614	1615
M33	C38	C40	C42	C44	C46	C48	C50	C52	C54	C56	C58	C60	C62	C64	C66	C68	C70	C72	1616	1617
M34	C74	C76	C78	C80	C82	C84	C86	C88	C90	C92	C94	C96	C98	C100	C102	C104	C106	C108	C110	C112
M35	C120	C122	C124	C126	C128	C130	C132	C134	C136	C138	C140	C142	C144	C146	C148	C150	C152	C154	C156	C158
M36	C160	C162	C164	C166	C168	C170	C172	C174	C176	C178	C180	C182	C184	C186	C188	C190	C192	C194	C196	C198
M37	C200	C202	C204	C206	C208	C210	C212	C214	C216	C218	C220	C222	C224	C226	C228	C230	C232	C234	C236	C238
M38	C240	C242	C244	C246	C248	C250	C252	C254	C256	C258	C260	C262	C264	C266	C268	C270	C272	C274	C276	C278
M39	C280	C282	C284	C286	C288	C290	C292	C294	C296	C298	C300	C302	C304	C306	C308	C310	C312	C314	C316	C318
M40	C320	C322	C324	C326	C328	C330	C332	C334	C336	C338	C340	C342	C344	C346	C348	C350	C352	C354	C356	C358

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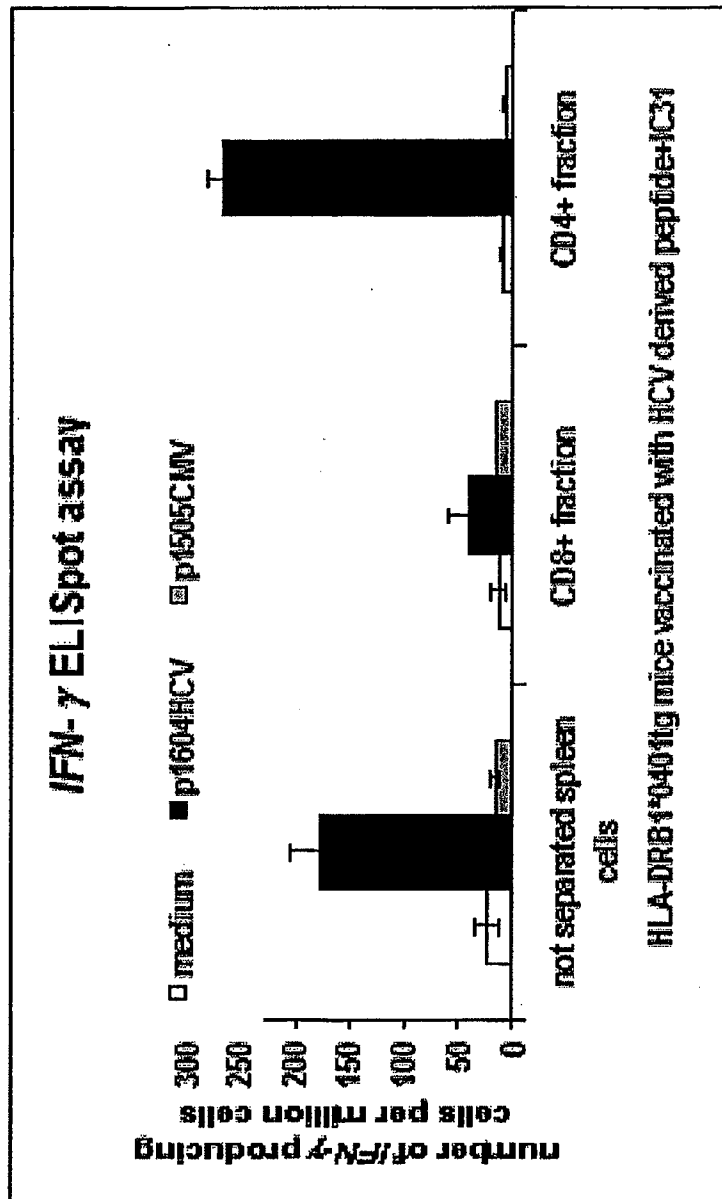


Fig.8

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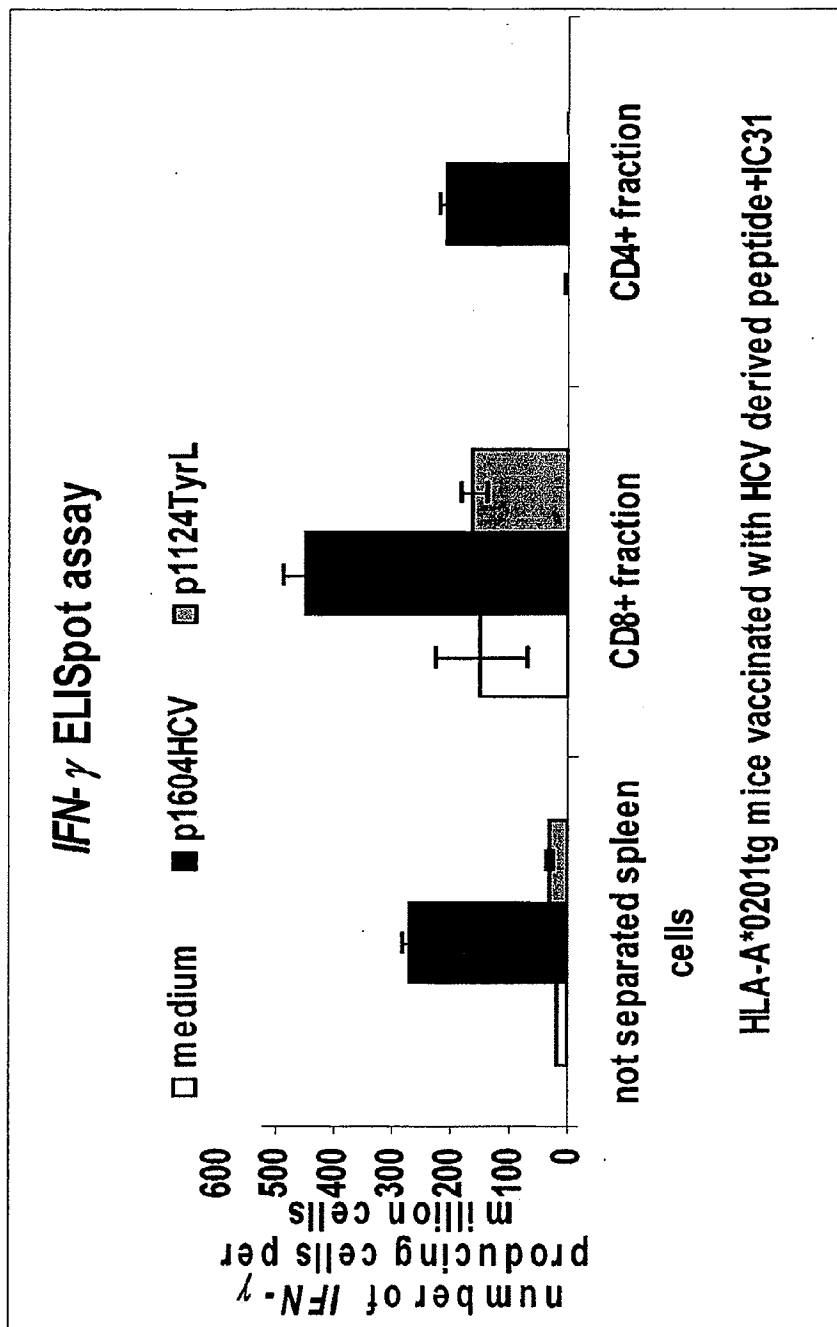


Fig.9

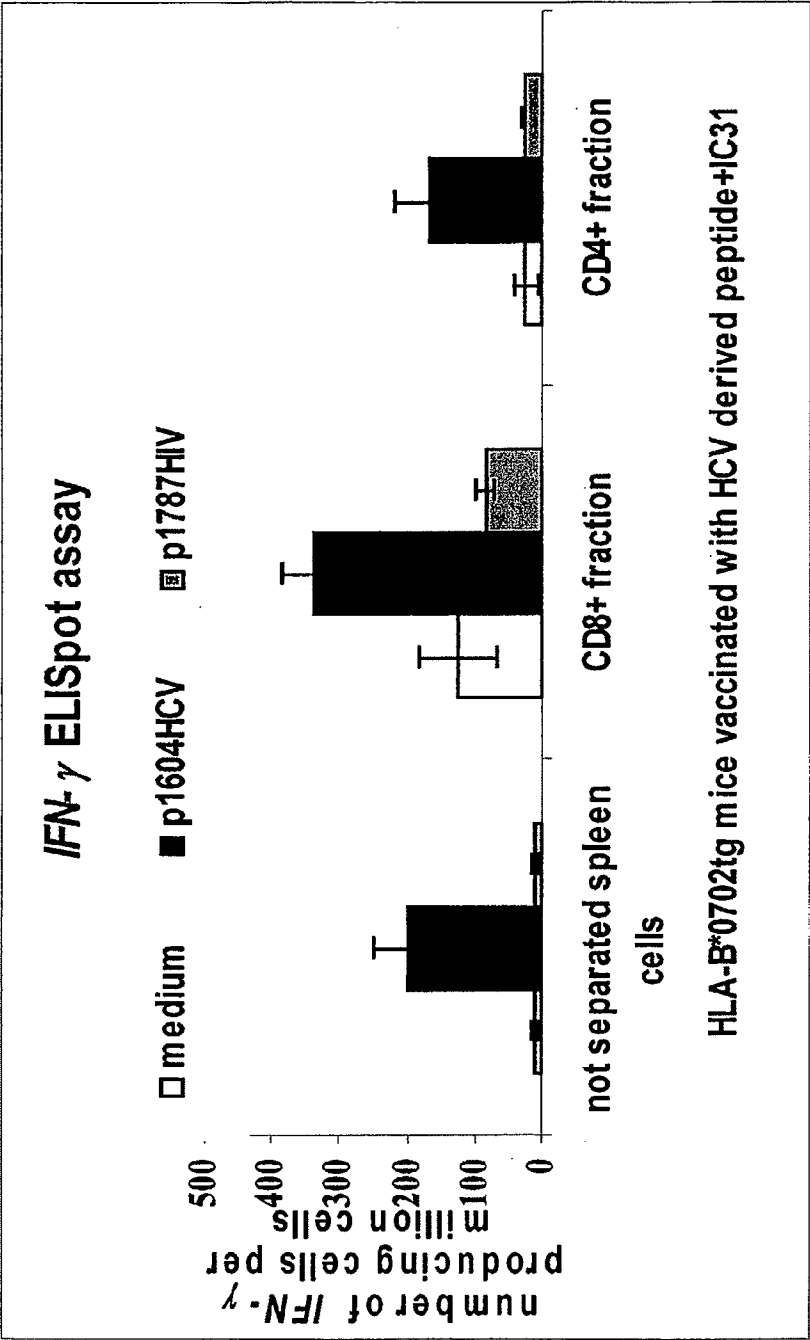


Fig.10